Evaluation of Bio-Oil produced from Fast Pyrolysis of Lignocellulosic Biomass as Carbon Source for Bacterial Bioconversion

DISSERTATION to obtain the
DOCTORAL DEGREE OF NATURAL SCIENCES
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Faculty of Natural Sciences of the University of Hohenheim,
Institute of Food Science and Biotechnology,
Department of Bioprocess Engineering

by

Stefanie Arnold, M.Sc.
from Balingen, Baden-Württemberg, Germany

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Dean:	Prof. Dr. rer. nat. Uwe Beifuß
1st reviewer / examiner:	Prof. Dr.-Ing. Rudolf Hausmann
2nd reviewer / examiner:	Prof. Dr. rer. nat. Christoph Syldatk
3rd examiner:	Prof. Dr. rer. nat. Johannes Gescher

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You never fail until you stop trying.

- Albert Einstein -

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List of Publications

The research work framing this thesis was conducted under supervision of Prof. Dr.-Ing. Rudolf Hausmann at the University of Hohenheim, Institute of Food Science and Biotechnology, Department of Bioprocess Engineering between 2015 and 2018.

Parts of this work have been published in peer-reviewed journals and excerpts of this research have been presented at international conferences as oral or poster presentations:

Literature Review Article

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Biotechnological perspectives of pyrolysis oil for a bio-based economy
  Trends in Biotechnology 2017, 35(10), pp. 925 - 936
  https://doi.org/10.1016/j.tibtech.2017.06.003

Original Research Articles

- Arnold, S., Moss, K., Dahmen, N., Henkel, M., Hausmann, R.
  Pretreatment strategies for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass
  GCB Bioenergy 2019, 11(1), pp. 181 - 190
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- Arnold, S., Tews, T., Kiefer, M., Henkel, M., Hausmann, R.
  Evaluation of small organic acids present in fast pyrolysis bio-oil from lignocellulose as feedstocks for bacterial bioconversion
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- Arnold, S., Henkel, M., Wanger, J., Wittgens, A., Rosenau, F., Hausmann, R.
  Heterologous rhamnolipid biosynthesis by *P. putida* KT2440 on bio-oil derived small organic acids and fractions
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Oral Presentation

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Evaluation of pyrolysis oil as a platform substrate for the bioeconomy
  1st Status Seminar of the Bioeconomy Research Program Baden-Württemberg, October 29th, 2015, Stuttgart, Germany

Poster Presentations

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Pyrolysis oil – A renewable resource as an alternative carbon source for microbial conversion?
  2nd Status Seminar of the Bioeconomy Research Program Baden-Württemberg, October 13th, 2016, Stuttgart, Germany

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Opportunities and challenges of industrial biotechnology for complete utilization of pyrolysis oil
  DECHEMA Himmelfahrtstagung 2017 – Models for Developing and Optimising Biotech Production, May 22nd - 24th, 2017, Neu-Ulm, Germany

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Evaluation of pyrolysis oil as an alternative microbial feedstock for a bio-based economy
  2nd Bioeconomy Congress Baden-Württemberg, September 12th - 13th, 2017, Stuttgart, Germany

- Arnold, S., Moss, K., Henkel, M., Hausmann, R.
  Evaluation of pyrolysis oil as an alternative microbial feedstock for a bio-based economy
  4th Status Seminar of the Bioeconomy Research Program Baden-Württemberg, January 8th, 2019, Stuttgart, Germany

- Merkel, M., Arnold, S., Henkel, M., Hausmann, R.
  Towards: Utilization of process waters from thermochemical conversion processes for continuous production of biosurfactants
  Biosurfactants 2019, September 25th - 27th, 2019, Stuttgart, Germany

Additional Publications (not directly related to this thesis)

- Arnold, S., Rodriguez-Uribe, A., Misra, M., Mohanty, A.K.
  Slow pyrolysis of bio-oil and studies on chemical and physical properties of the resulting new bio-carbon
  Original Research Article in *Journal of Cleaner Production* 2018, 172, pp. 2748 - 2758, https://doi.org/10.1016/j.jclepro.2017.11.137

- Arnold, S., Rodriguez-Uribe, A., Misra, M., Mohanty, A.K.
  Slow pyrolysis of bio-oil and studies on chemical and physical properties of the resulting new bio-carbon
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Authors' Contributions

This work has been partially published previously with the knowledge and approval of the supervisor Prof. Dr.-Ing. Rudolf Hausmann. The scientific work presented in this thesis was partially conducted in cooperation with co-authors from the University of Hohenheim, Karlsruhe Institute of Technology and Ulm University.

- **Biotechnological perspectives of pyrolysis oil for a bio-based economy**
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  SA performed literature research, created the figures, conceived and drafted the manuscript. All authors contributed to this article and proof-read the manuscript.

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  SA planned and executed the experiments, collected data, interpreted the results, created the graphs, figures and tables and drafted the manuscript. KM contributed to scientific discussions and proof-read the manuscript. MH contributed to conception and design of the study, interpretation of the experiments and proof-read the manuscript. RH supervised the project, proof-read the manuscript and provided input for the design of the study.

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  SA planned and executed the experiments, collected data, interpreted the results, created the graphs, figures and tables and drafted the manuscript. TT and MK performed part of the experiments and collected and evaluated corresponding data. MH contributed to conception and design of the study and interpretation of the experiments. RH supervised the project and provided input for the design of the study. All authors read and approved the final version of the manuscript.

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  SA planned and executed the experiments, collected data, created the graphs and drafted the manuscript. MH designed and planned the experiments, created the graphs and drafted the manuscript. JW performed part of the experiments and collected and evaluated corresponding data. AW and FR generated the plasmid pSynPro8_rhlAB and contributed to interpretation of the experiment. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

Abstract

Scarcity of fossil resources, climate change and growing world population demand the transition from a fossil-based economy towards a bioeconomy – a knowledge-based strategy which relies on the efficient and sustainable integration of bio-based resources into value-added process chains. As lignocellulosic biomass is an abundant renewable resource which does not directly compete with food and feed, its deployment in biorefineries is of special interest for a sustainable bioeconomy. Owing to its compact and complex structure, suitable conversion techniques need to be selected. Combinations of thermochemical and biochemical conversion technologies are considered to be a promising approach regarding a fast and efficient conversion of lignocellulosic biomass into value-added products.

Bio-oil derived from fast pyrolysis of lignocellulosic biomass is a complex mixture and composed of water and a wide variety of organic components. Among these components pyrolytic sugars and small organic acids are particularly interesting as potential carbon sources for microbial processes. However, bio-oil also comprises many unidentified substances, as well as components which are known to display adverse effects on microbial growth. To evaluate the potential and challenges of bio-oil as an alternative and sustainable carbon source for bacterial bioconversion this thesis was divided into three parts (Figure 1).

In **Part I** different pretreatment strategies were applied and evaluated regarding their effect on stability and detoxification of bio-oil fractions. For this purpose, the organic solvent tolerant bacterial strain *Pseudomonas putida* KT2440 was applied as a reference system and cultivated on different pretreated bio-oil fractions. It was shown that solid phase extraction is a suitable tool to obtain bio-oil fractions with significantly increased stability along with less inhibitory substances.

Part II is focused on the evaluation of small organic acids mainly present in bio-oil with respect to their suitability as feedstock for bacterial growth. Four biotechnological production hosts *Escherchia coli*, *Pseudomonas putida*, *Bacillus subtilis* and *Corynebacterium glutamicum* were cultivated on different concentrations of

acetate, mixtures of small organic acids, as well as pretreated bio-oil fractions as carbon source for their growth. Results reveal that *P. putida*, as well as *C. glutamicum* metabolizes acetate – the major small organic acid generated during fast pyrolysis of lignocellulosic biomass – as sole carbon source over a wide concentration range and grow on mixtures of small organic acids present in bio-oil. Moreover, both strains show a distinct potential to tolerate inhibitory substances within bio-oil.

Part III describes the growth behavior of a genetically engineered, non-pathogenic bacterium *Pseudomonas putida* KT2440 and its simultaneous heterologous production of rhamnolipid biosurfactants on bio-oil derived small organic acids and pretreated fractions. Results suggest that both maximum achievable productivities and substrate-to-biomass yields are in a comparable range for glucose, acetate, as well as the mixture of acetate, formate and propionate. Similar yields were obtained for a pretreated bio-oil fraction, although with significantly lower titers.

In conclusion, this thesis shows that microbial valorization of bio-oil is a challenging task due to its highly complex and variable composition, as well as its adverse effects on microbial growth and issues with analytical procedures. This work depicts a proof of concept by outlining a potential biorefinery route for microbial valorization of pretreated bio-oil and its unexploited side streams. It provides a step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks into value-added products and thus contributes to establishing bioprocesses within a future bioeconomy.

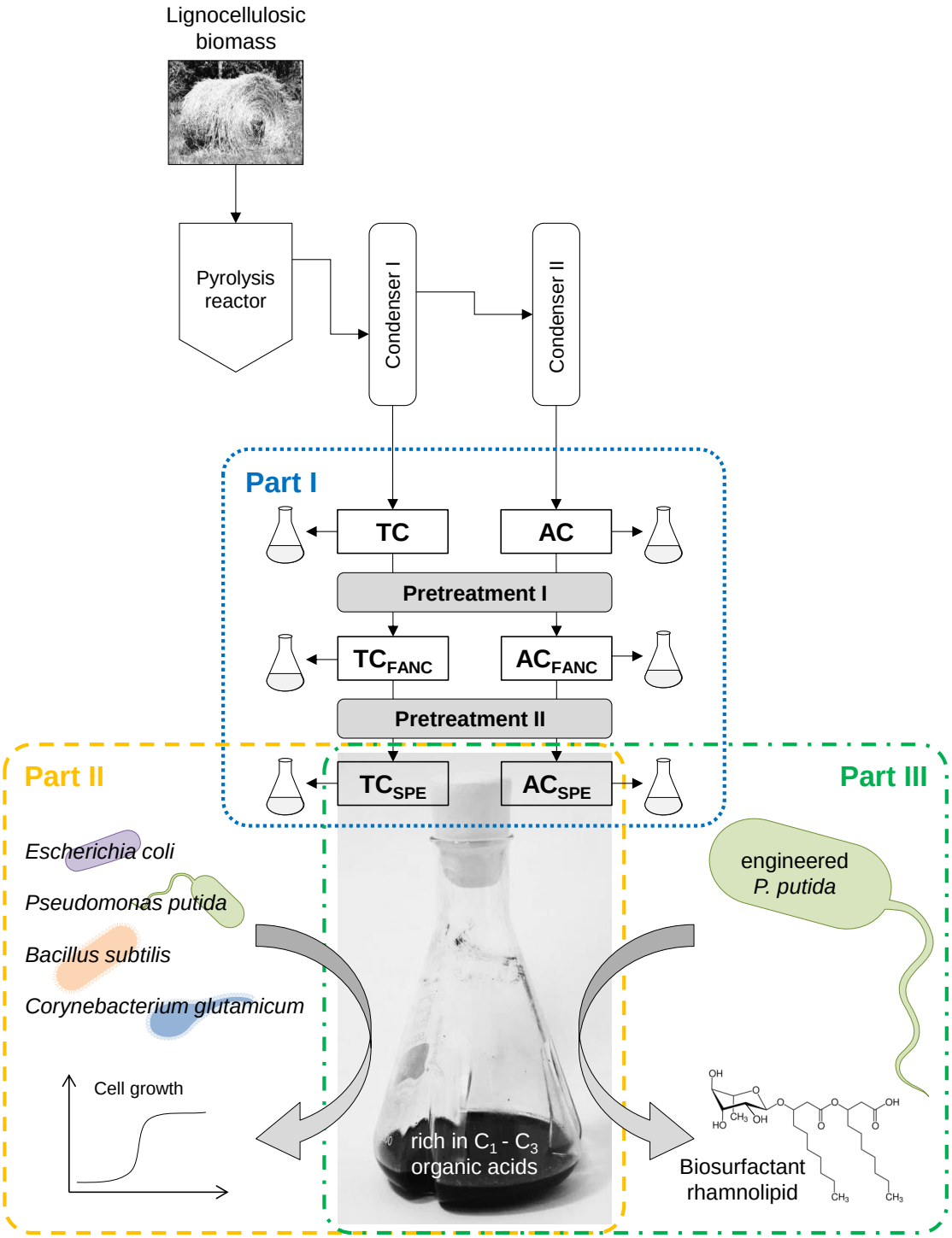

Figure 1: Graphical Abstract – The three major parts of this thesis

Zusammenfassung

Verknappung fossiler Ressourcen, der Klimawandel und eine wachsende Weltbevölkerung fordern den Wandel von einer auf fossilen Rohstoffen basierenden Wirtschaft hin zu einer Bioökonomie – einer wissensbasierten Strategie, die auf der effizienten und nachhaltigen Integration biobasierter Ressourcen in Wertschöpfungsketten beruht. Da es sich bei lignocellulosehaltiger Biomasse um eine reichlich vorhandene, nachwachsende und nicht direkt mit Lebens- und Futtermitteln konkurrierende Ressource handelt, ist ihre Verwendung in Bioraffinerien für eine nachhaltige Bioökonomie von besonderem Interesse. Aufgrund ihrer kompakten und komplexen Struktur müssen geeignete Konversionstechniken ausgewählt werden. Für eine schnelle und effiziente Umwandlung von lignocellulosehaltiger Biomasse in höherwertige Produkte sind vor allem Kombinationen aus thermochemischen und biochemischen Konversionstechnologien ein vielversprechender Ansatz.

Das durch Schnell-Pyrolyse von lignocellulosehaltiger Biomasse erzeugte Bio-Öl ist ein komplexes Gemisch, das aus Wasser und einer Vielzahl organischer Komponenten besteht. Dabei zählen vor allem pyrolytische Zucker und niedermolekulare organische Säuren als besonders vielversprechende Kohlenstoffquellen für mikrobielle Prozesse. Bio-Öl umfasst jedoch auch viele unidentifizierte Substanzen sowie Komponenten, deren negative Auswirkungen auf das mikrobielle Wachstum bekannt sind. Um das Potenzial und die Herausforderungen des Bio-Öls als alternative und nachhaltige Kohlenstoffquelle für die bakterielle Biokonversion zu bewerten, wurde diese Arbeit in drei Teile gegliedert (Figure 1).

In **Part I** wurden verschiedene Vorbehandlungsstrategien angewandt und hinsichtlich ihres Einflusses auf die Stabilität und Entgiftung von Bio-Öl-Fraktionen bewertet. Hierfür wurde der gegenüber organischen Lösungsmitteln tolerante Bakterienstamm *Pseudomonas putida* KT2440 als Referenzsystem eingesetzt und auf verschiedenen vorbehandelten Bio-Öl-Fraktionen kultiviert. Es wurde gezeigt, dass die Festphasenextraktion ein geeignetes Werkzeug ist, um stabile Bio-Öl-Fraktionen mit weniger hemmenden Substanzen zu erhalten.

Part II befasst sich mit der Bewertung im Bio-Öl vorkommender niedermolekularer organischer Säuren hinsichtlich ihrer Eignung als Rohstoff für bakterielles Wachstum. Vier biotechnologische Produktionsstämme *Escherchia coli*, *Pseudomonas putida*, *Bacillus subtilis* und *Corynebacterium glutamicum* wurden auf unterschiedlichen Konzentrationen von Acetat, Mischungen niedermolekularer organischer Säuren, sowie vorbehandelten Bio-Öl-Fraktionen als Kohlenstoffquelle für deren Wachstum kultiviert. Die Ergebnisse zeigen, dass sowohl *P. putida* als auch *C. glutamicum* Acetat – die wichtigste organische Säure, die bei der Schnell-Pyrolyse von lignocellulosehaltiger Biomasse entsteht – über einen großen Konzentrationsbereich hinweg als einzige Kohlenstoffquelle verstoffwechseln und auf Mischungen niedermolekularer organischer Säuren, welche im Bio-Öl vorkommen, wachsen. Darüber hinaus zeigen beide Stämme ein eindeutiges Potenzial inhibitorische Substanzen im Bio-Öl zu tolerieren.

Part III beschreibt das Wachstumsverhalten eines gentechnisch veränderten, nicht pathogenen Bakteriums *Pseudomonas putida* KT2440 und dessen gleichzeitige heterologe Produktion von Rhamnolipid-Biotensiden auf niedermolekularen organischen Säuren und vorbehandelten Bio-Öl-Fraktionen. Die Ergebnisse zeigen, dass sowohl die maximal erreichbaren Produktivitäten als auch die Substrat-Biomasse-Ausbeuten für Glucose, Acetat sowie eine Mischung aus Acetat, Format und Propionat in einem vergleichbaren Bereich liegen. Ähnliche Ausbeuten wurden bei einer vorbehandelten Bio-Öl-Fraktion erzielt, wenn auch mit signifikant niedrigeren Titern.

Zusammenfassend zeigt diese Arbeit, dass die mikrobielle Verwertung von Bio-Öl aufgrund seiner hochkomplexen und variablen Zusammensetzung, seiner nachteiligen Auswirkungen auf das mikrobielle Wachstum und Schwierigkeiten gegenüber analytischen Verfahren eine herausfordernde Aufgabe darstellt. Die Arbeit beschreibt einen Machbarkeitsnachweis, indem ein möglicher Bioraffinerie-Weg für die mikrobielle Verwertung von vorbehandeltem Bio-Öl und seinen ungenutzten Nebenströmen aufgezeigt wird. Es stellt einen Schritt auf der Suche nach geeigneten Bakterienstämmen zur biologischen Umwandlung von lignocellulosebasierten Rohstoffen in höherwertige Produkte dar und trägt somit zur Etablierung von Bioprozessen in einer zukünftigen Bioökonomie bei.

Table of Contents

Chapter **1**

General Introduction

1.1 Towards a Sustainable Bioeconomy

> *"**Bioeconomy** [also bio-based economy] encompasses the production of renewable biological resources and the conversion of these resources and waste streams into value-added products, such as food, feed, bio-based products and bioenergy."*
>
> (European Commission 2012)

The world is facing many challenges, from population growth, food shortage to climate change and depletion of fossil resources. Hence, many industrialized countries have begun the transition from a fossil-based economy towards a **bioeconomy** – a knowledge-based strategy which relies on the efficient and sustainable integration of **bio-based resources** into value-added process chains (Figure 2). A bio-based value-added process chain encompasses the primary production of bio-based resources, their processing into value-added products, and their commercialization (Lewandowski 2018). This involves a variety of sectors including agriculture, forestry, fisheries, and aquaculture for primary production, as well as food, pulp and paper industries, together with parts of chemical, biotechnological and energy industries for processing of bio-based resources (European Commission 2012). Thus, bioeconomy is a highly innovative and interdisciplinary field. As the basic idea of a bio-based economy is processing of bio-based resources into a variety of different value-added products, **biorefineries** are an integral part of all bioeconomy value-added process chains (Figure 2). Biorefineries are facilities which aim at an optimal utilization of bio-based resources by integrating intelligent combination and cascading of different **biomass** processing technologies and equipment for a co-production of food, feed, bio-based products (materials, chemicals), and bioenergy (power, heat, fuels) (de Jong and Jungmeier 2015).

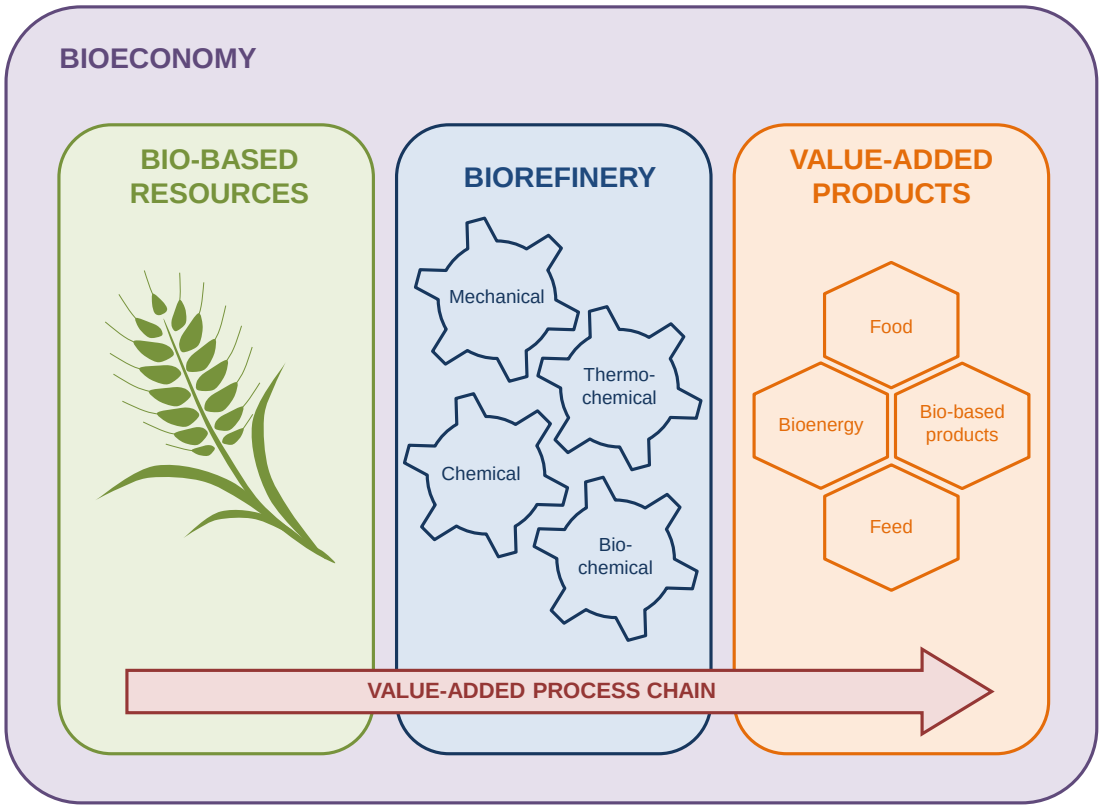

Figure 2: Bioeconomy – From bio-based resources towards value-added products

> *"**Bio-based resources** are all resources containing non-fossil, organic carbon, recently (< 100 years) derived from living plants, animals, algae, microorganisms or organic waste streams. These are summarized in the term **biomass**."*
>
> (Zörb and Lewandowski 2018)

Common biomass processing technologies are classified into mechanical (e.g., size reduction, extraction, fractionation, separation), thermochemical (e.g., combustion, pyrolysis, gasification, liquefaction), chemical (e.g., acid hydrolysis, synthesis, esterification), and/or biochemical (e.g., fermentation, anaerobic digestion, enzymatic conversion) conversion processes (Hendriks and Zeeman 2009, FitzPatrick *et al.* 2010, Menon and Rao 2012, Isikgor and Becer 2015, Maurya *et al.* 2015, Kumar and Sharma 2017). For a resource-efficient and profitable value-added process chain a biorefinery should be a close-to-zero-waste process by making use of recycling options and employing cascaded conversion processes for a fully utilization of all product and side streams.

> *"**Biorefinery** is the sustainable processing of biomass into a spectrum of marketable products and energy."*
>
> (de Jong and Jungmeier 2015)

The main focus has to be placed on issues related to land use, protection of the environment, climate, and biodiversity, as well as food security to achieve a successful transition towards a sustainable bioeconomy. While heat and electrical power can be provided by other renewable resources such as solar, wind, hydro or geothermal energy, biomass is the sole renewable resource for the production of bio-based products. Therefore, it is important that biomass is primarily used for the production of materials and chemicals, and only for energetic purposes if the biorefinery integrates both material and energy uses of biomass. Furthermore, biomass of plant origin, which does not directly compete with food or feed, is considered as a bio-based resource for biorefineries and of special interest for a sustainable bioeconomy (Arevalo-Gallegos *et al.* 2017, da Silva *et al.* 2012).

1.2 Lignocellulosic Biomass: A Sustainable Non-Food Bio-Based Resource

Lignocellulosic biomass is the most abundant bio-based resource. Lignocellulose is the major building block of plant cell walls and consists of the three biopolymers cellulose (glucose polymer), hemicellulose (polymer rich in pentose sugars), and lignin (cross-linked phenolic polymer) (Figure 3). Depending on plant species and growth conditions the structure and composition of these biopolymers can vary greatly (Table 1). In general, lignocellulosic biomass comprises about 25 - 55 % cellulose, 17 - 40 % hemicellulose, and 10 - 35 % lignin (Anwar *et al.* 2014). Cellulose is an unbranched linear homopolysaccharide of up to 10,000 β-1,4-linked D-glucose molecules. In contrast, hemicellulose is a branched, amorphous heteropolymer containing hexoses (D-glucose, D-mannose and D-galactose), pentoses (D-xylose and L-arabinose) and sugar acids (e.g., glucuronic acid, galacturonic acid). Lignin is a highly branched and complex three-dimensional polyphenolic macromolecule mainly derived from three alcohol monomers p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Cellulose is connected with hemicellulose and lignin by hydrogen bonds, whereas hemicellulose and lignin are connected with each other by covalent bonds. This tight

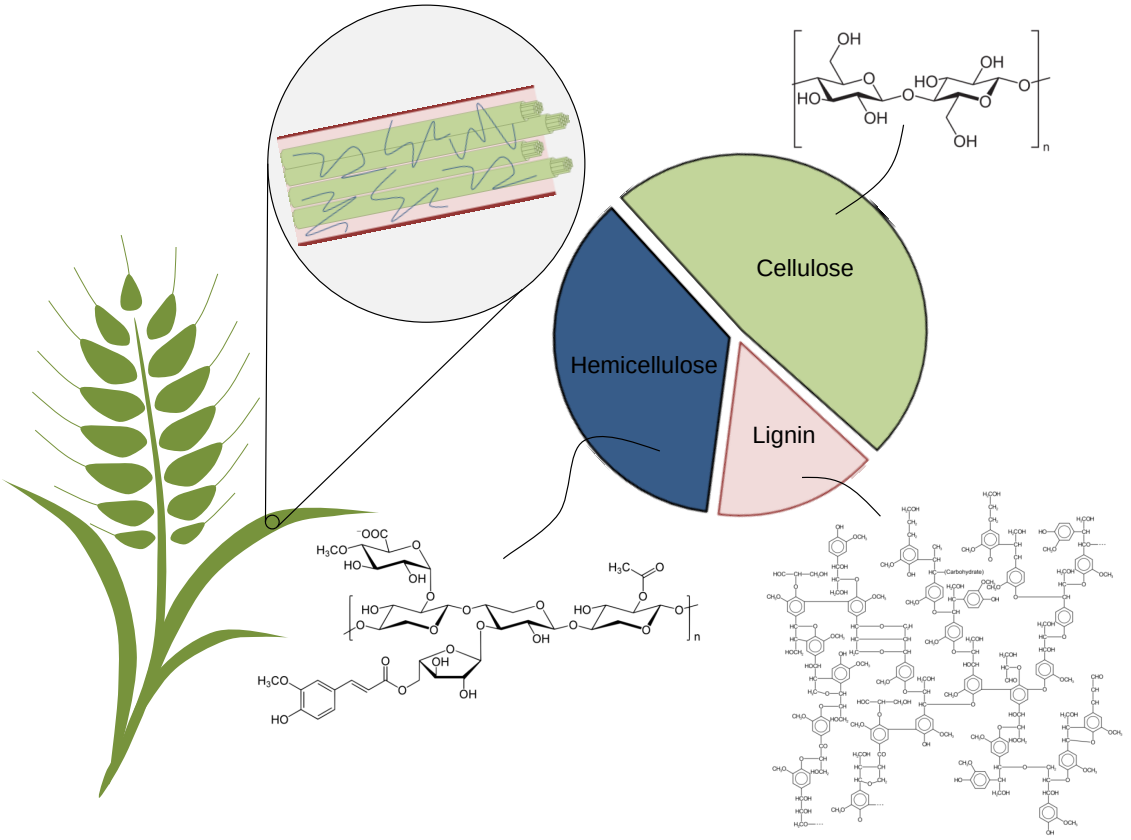

Figure 3: Composition of lignocellulosic biomass

and complex spatial structure confers plants their stability and resistance against microbial and mechanical forces.

> ***Lignocellulosic biomass*** *refers to plant biomass (e.g., rice straw, wheat straw, corn stover, bagasse, wood wastes, agricultural residues) and is composed of cellulose, hemicellulose, and lignin.*
>
> (Kumar and Sharma 2017)

As lignocellulosic biomass is not directly linked to food or feed supply, its deployment in biorefineries is of special interest for a sustainable bioeconomy. However, utilization of lignocellulosic biomass also entails some drawbacks which are mainly associated with its complex and recalcitrant composition, diversity and formation of inhibitory and toxic by-products during its conversion. Depending on lignocellulosic biomass feedstock and requirements, appropriate conversion techniques need to be selected. These techniques may be applied individually, in a particular order or in combination for an efficient degradation of the highly complex and diverse biomass into its constituents and converting the fullest possible extent into several

Table 1: Composition of various lignocellulosic biomasses (Anwar *et al.* 2014)

Lignocellulosic biomass	Cellulose [%]	Hemicellulose [%]	Lignin [%]
hardwood	40 - 55	24 - 40	18 - 25
softwood	45 - 50	25 - 35	25 - 35
corn stover	38	26	19
rice straw	38	24	18
grasses	25 - 40	25 - 40	10 - 30
wheat straw	29 - 35	26 - 32	16 - 21
bagasse	55	17	23

product streams. As mentioned in section 1.1, different biomass processing technologies have been developed. Beside complex and multi-step methods, thermochemical processes can convert a large variety of biomass feedstocks directly into value-added products.

1.3 Thermochemical Conversion of Biomass via Pyrolysis*

Fast and direct conversion of whole biomass into value-added products can be achieved by thermochemical processes such as **pyrolysis**. During the pyrolysis process, biomass is subjected to a variety of physical and chemical alterations resulting always in three different product streams: a carbon-rich solid (bio-char), an energy-rich liquid (pyrolysis oil or bio-oil), and a combustible, non-condensable gas (syngas) (Bridgwater 1996, Bridgwater *et al.* 1999, Jarboe *et al.* 2011). The relative proportions of the pyrolysis products depend on the feedstock and process conditions including temperature, heating rate, and residence time (Bridgwater 1996). Based on the adjusted process parameters three different modes of pyrolysis processes can be classified

> ***Pyrolysis*** *(from Greek pyro – fire; lysis – decomposition) is a thermochemical decomposition of organic materials in the absence of oxygen.*
>
> (Bridgwater 2004, Jarboe *et al.* 2011)

*Parts of this section are based on the publication "Biotechnological Perspectives of Pyrolysis Oil for a Bio-Based Economy" (see section 1.4)

maximizing the yield of a desired product: carbonization, fast pyrolysis, and gasification (Bridgwater 2004). While carbonization is characterized by low temperatures and very long residence times resulting in solid bio-char as the main product, fast pyrolysis leads to maximum yields of liquid bio-oil by treating biomass at moderate temperatures and short residence times. Gasification, which includes a pyrolysis step, is performed at high temperatures and long residence times to obtain high yields of syngas.

> ***Bio-oil**, also known as pyrolysis oil or bio-crude, is mainly produced by fast pyrolysis of biomass. It is a complex, dark brown, acidic, viscous, and energy-rich liquid with a distinctive smoky odor.*
>
> (Czernik and Bridgwater 2004)

As fast pyrolysis aims at maximizing the yield of an easy storable and transportable energy-rich liquid, the production of **bio-oil** is considered as one of the promising ways to convert biomass into building block chemicals for transportation fuels and other value-added products (Bridgwater 1996, Bridgwater *et al.* 1999, Czernik and Bridgwater 2004, Jacobson *et al.* 2013). Different pyrolysis reactor configurations have already been explored to optimize liquid production from biomass. The following mentioned reactor types are the most popular ones: (i) fluidized-bed reactor, (ii) ablative reactor, and (iii) vacuum pyrolysis reactor (Bridgwater *et al.* 1999). Especially fluidized-bed reactor configurations are in particular focus due to their convenient handling and easy implementability in rural decentral facilities. For an efficient fast pyrolysis process (Figure 4) the biomass first has to be dried to a moisture of $< 10\,\%$ and grinded to small particles of $< 2\,\text{mm}$, before it is fed to the pyrolysis reactor where the fast pyrolysis takes place at temperatures around $500\,^{\circ}\text{C}$ with a heat carrier (e.g., sand) (Bridgwater *et al.* 1999). The biomass is rapidly heated in the absence of oxygen and converted into a gaseous form. The pyrolysis vapor passes several cyclones to remove char before entering the condenser, where it is rapidly quenched and converted into a dark brown liquid which is commonly named bio-oil or pyrolysis oil (Bridgwater *et al.* 1999). The remaining non-condensable gases and char are used to provide the process heat, so that no waste streams are generated.

The complex mixture of bio-oil comprises both polar and non-polar chemicals with various molecular-weight mainly derived from rapid and simultaneous depolymerization and fragmentation of the three buildings blocks of lignocellulose:

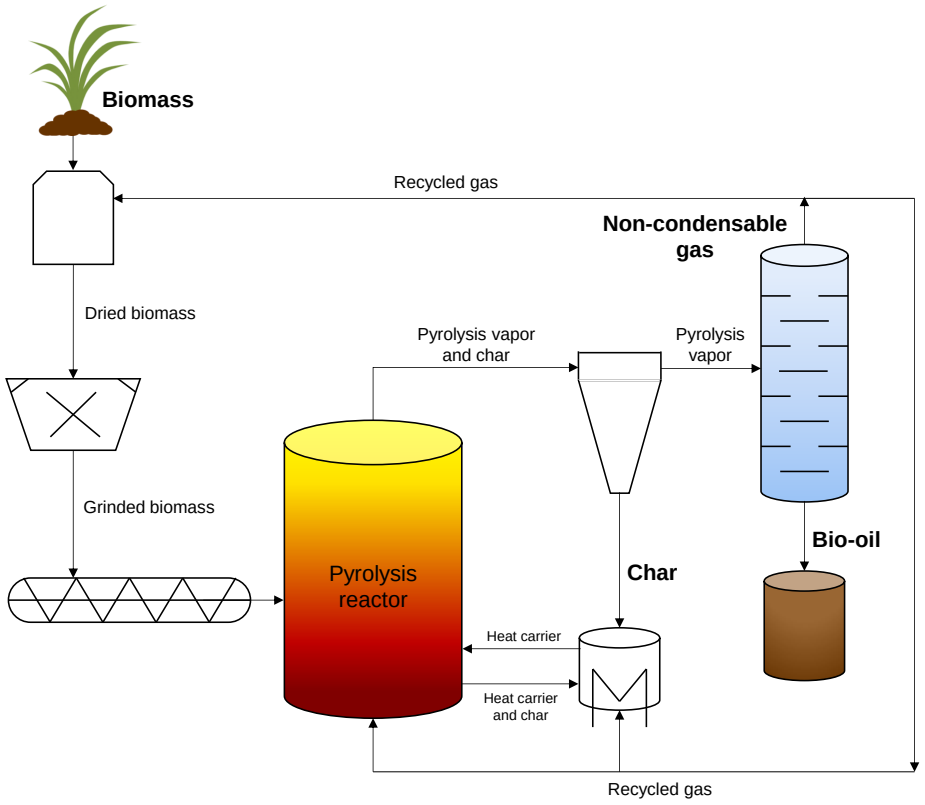

Figure 4: Overview of a fast pyrolysis process

cellulose, hemicellulose, and lignin (Czernik and Bridgwater 2004). Depending on the biomass and pyrolysis conditions the chemical composition varies greatly (Bridgwater *et al.* 1999) and complete chemical characterization is not practical. However, all bio-oils derived from biomass elementally resemble the source biomass, both consisting of the main elements carbon (32 - 49 wt%), hydrogen (6.9 - 8.6 wt%), oxygen (44 - 60 wt%), nitrogen (0 - 0.2 wt%) and sulfur (0 - 0.05 wt%) (Oasmaa and Czernik 1999). Bio-oils are principally composed of water (15 - 30 wt%) (Oasmaa and Czernik 1999, Czernik and Bridgwater 2004) and a wide variety of organic components including organic acids (e.g., acetic acid, formic acid, propionic acid), carbonyl and hydroxycarbonyl compounds (aldehydes and ketones), sugars and anhydrosugars (e.g., levoglucosan), alcohols, miscellaneous oxygenates (e.g., hydroxyacetaldehyde, acetol), as well as phenolic lignin and aromatic compounds (Piskorz *et al.* 1988) (Figure 5). However, nearly a third of the mass fraction cannot be identified.

The presence of many organic acids within the bio-oil leads to a low pH of 2 - 4 (Oasmaa and Czernik 1999, Bardalai and Mahanta 2015). The large amount of incorporated oxygen is responsible for chemical instability and leads to a wide

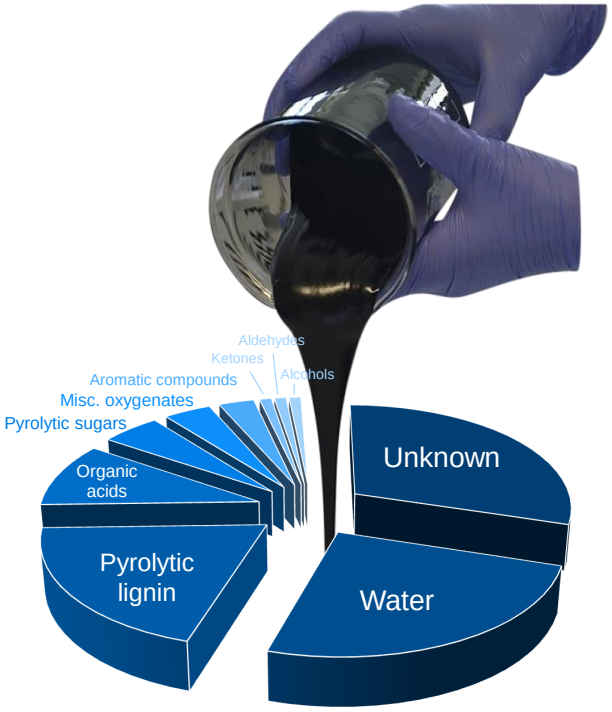

Figure 5: Bio-oil and its composition

range of both boiling point temperature and viscosity (Czernik and Bridgwater 2004). Typically, bio-oils are single phase liquids with a density of 1.1 - 1.3 kg/m^3 (Oasmaa and Czernik 1999). During storage of bio-oils derived from pyrolysis of ash-rich or high-moisture feedstocks such as annual plants or nonwoody biomass spontaneous phase separation can occur resulting in a two-phase bio-oil consisting of a viscous organic phase and an aqueous phase (Oasmaa and Czernik 1999, Demirbas 2007, Dahmen *et al.* 2012, Bardalai and Mahanta 2015).

Bio-oil has already been deployed successfully in furnaces, boilers, or gas turbines to generate heat and power or upgraded to bio-fuels. However, properties such as high corrosiveness, high viscosity, instability, low heating values, highly oxidative character, complexity, and diversity make its application very challenging. Several strategies have been proposed to improve quality and stability of bio-oil (Oasmaa and Czernik 1999, Demirbas 2007, Westerhof *et al.* 2010, Bridgwater 2012). An option to control the quality of bio-oils is fractionation of vapors produced during fast pyrolysis by using a serial condenser train for the production of different liquid fractions with a more defined composition improved for different applications (Westerhof *et al.* 2011, Pollard *et al.* 2012, Lian *et al.* 2012, Pfitzer

et al. 2016). An approach for converting bio-oil fractions into highly selected value-added products is their utilization as carbon sources in biotechnological processes.

1.4 Biotechnological Perspectives of Pyrolysis Oil for a Bio-Based Economy

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Opinion

Biotechnological Perspectives of Pyrolysis Oil for a Bio-Based Economy

Stefanie Arnold,[1] Karin Moss,[1] Marius Henkel,[1,*] and Rudolf Hausmann[1]

Lignocellulosic biomass is an important feedstock for a potential future bio-based economy. Owing to its compact structure, suitable decomposition technologies will be necessary to make it accessible for biotechnological conversion. While chemical and enzymatic hydrolysis are currently established methods, a promising alternative is provided by fast pyrolysis. The main resulting product thereof, referred to as pyrolysis oil, is an energy-rich and easily transportable liquid. Many of the identified constituents of pyrolysis oil, however, have previously been reported to display adverse effects on microbial growth. In this Opinion we discuss relevant biological, biotechnological, and technological challenges that need to be addressed to establish pyrolysis oil as a reliable microbial feedstock for a bio-based economy of the future.

Pyrolysis Oil in a Bio-Based Economy

For a future **bio-based economy** (see Glossary), efficient and economic methods of converting renewable resources into valuable bulk products need to be established. Biorefinery concepts generally rely on maximum valorization of **lignocellulosic materials** including non-foods such as wood residues and agricultural wastes.

For efficient biotechnological conversion of lignocellulosic materials, fractionation and decomposition technologies are necessary to produce monomers or building blocks suitable for microbial metabolism. Several methods for the decomposition of lignocellulose have been developed, including chemical, enzymatic, biological, and thermal technologies. The established procedure of lignocellulose saccharification consists of polysaccharide hydrolysis steps coupled with various fractionation methods resulting in sugar monomers. This work focuses on a non-established alternative for depolymerization and biotechnological utilization of lignocellulosic material: pyrolysis (Box 1).

Thermal processes such as **fast pyrolysis** are commonly referred to as economically and environmentally friendly methods. The aim of fast pyrolysis is to maximize, in a single step, the yield of an easily storable and transportable energy-rich liquid [1] – the so-called **pyrolysis oil**. As part of the process, solid and gaseous components such as bio-char and syngas are also formed (Box 1). Depending on the biomass and pyrolysis conditions the chemical composition varies greatly and complete chemical characterization is not practical (Box 2). However, all pyrolysis oils derived from biomass elementally resemble the source biomass and consist of the main elements carbon (32–49 wt%), hydrogen (6.9–8.6 wt%), oxygen (44–60 wt%), nitrogen (0–0.2 wt%), and sulfur (0–0.05 wt%) [2]. Pyrolysis oils are principally composed of water (15–30 wt%) and a wide variety of organic components. These

Trends

Lignocellulosic biomass, the most abundant renewable resource, is a substantial feedstock for a future bio-based economy. Owing to its compact structure, suitable decomposition technologies for biotechnological use are required.

Fast pyrolysis converts lignocellulose into a liquid known as pyrolysis oil. The potential of this alternative and cheap carbon source is currently being assessed for biotechnological applications.

The properties of pyrolysis oil make its application very challenging. Several approaches have been reported for circumventing the restrictions imposed by pyrolysis oil. Tolerance engineering is an especially promising emerging trend.

First proofs of principle using pyrolysis oil as a substrate for industrially relevant bioproduction have been reported in which strains and process technologies are specifically tailored to converting this complex substrate.

[1]University of Hohenheim, Institute of Food Science and Biotechnology, Department of Bioprocess Engineering, Fruwirthstrasse 12, 70599 Stuttgart, Germany

*Correspondence: Marius.Henkel@uni-hohenheim.de (M. Henkel).

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Box 1. Pyrolysis Process, Products, and Current Applications

Pyrolysis is the direct thermochemical decomposition of organic materials in the absence of molecular oxygen to obtain a carbon-rich solid known as bio-char, an energy-rich liquid known as pyrolysis oil, and a combustible, non-condensable gas known as syngas [5]. The main advantage of pyrolysis is its feedstock flexibility; it is able to process many different raw materials including forest and agricultural residues [64], municipal wastes, and plastics [65]. The relative proportions of the pyrolysis products depend on the feedstock and process conditions including temperature, heating rate, and vapor residence time. Based on the adjusted process parameters, Bridgwater *et al*. classified three different modes of pyrolysis processes maximizing the yield of a desired product: carbonization, fast pyrolysis, and gasification [66].

Carbonization at low temperatures (300–400°C) and slow heating rates (5–50 K per minute) generates solid biochar. If the solid biomass is treated at moderate temperatures (400–600°C) and a fast heating rate (20 000 K per minute), maximum yields of liquid pyrolysis oil are achieved. This process is called fast pyrolysis. Gasification, which includes a pyrolysis step, is performed at high temperatures (800–900°C) to obtain high yields of a gaseous mixture of mainly H_2 and CO [67] – the so-called syngas.

Biochar is a solid decomposition product of lignocellulosic biomass, its main constitutes being carbon and a varying content of ash. It can be used as replacement for pulverized coal, or as a natural soil amendment to restore soil productivity. Recent research reported biochar as a promising colorant as well as a reactive or non-reactive filler when used as additive for plastics and composites [68].

Syngas is a combustible gas which can be used directly for generating heat and power. Furthermore, the Fischer–Tropsch process enables the catalytic conversion of syngas (CO and H_2) into a variety of liquid hydrocarbons, which in turn can be used as clean biofuels (e.g., green gasoline, diesel) [69]. A promising alternative to conventional applications is the production of fuels and chemicals through microbial fermentation of syngas (e.g., by *Clostridium ljungdahlii*) resulting in a mixture of ethanol and acetate [70,71].

include organic acids such as acetic, formic, and propionic acids; carbonyl and hydroxycarbonyl compounds such as aldehydes and ketones; sugars and anhydrosugars such as **levoglucosan** (Box 3); and phenolic compounds including pyrolytic lignin and phenols [3] (Figure 1). However, nearly a third of the mass fraction cannot be identified, possibly because analyses of the chemical composition of pyrolysis oil are mainly based on gas chromatography coupled with mass spectroscopy (GC/MS) [4], only allowing the determination of vaporizable molecules. It is assumed that many of the unidentified components are sugar oligomers and polymers [5,6].

The properties of pyrolysis oil, such as its high corrosiveness, high viscosity, instability, low heating values, highly oxidative character, complexity, and diversity make its application very challenging. Current reports of potential applications of pyrolysis oil mainly include its direct combustion in furnaces, boilers, and gas turbines to generate heat and power [7–9], its upgrading to potential transport fuels [10], or its utilization as a feedstock for the production of adhesives [11], resins [12], and fertilizers [13]. It is also used as a commercial food flavoring such as barbecue flavor [14]. Because of their high oxygen and water contents, pyrolysis oils exhibit heating values of about 13–18 MJ/kg, which is only 40–50% of that for hydrocarbon fuels [2]. Using fast pyrolysis as a technique for obtaining biofuels, process conditions may favor the formation of an energy-rich lipophilic fraction of pyrolysis oil containing low amounts of oxygen and high amounts of hydrogen. Phase separation of pyrolysis oil can be achieved by fractionated condensation or by spontaneous separation during storage (Box 2). Because of the chemical composition of lignocellulose, a process-inherent hydrophilic low-value fraction is formed. This fraction in principle comprises water, small organic acids, ketones, and anhydrosugars. The exact composition depends on the details of the process. In the value chain this fraction is a low-value side-product that compromises the utilization of pyrolysis products for energy generation.

Biotechnological processes are especially promising approaches for converting this highly complex substrate mixture into products. As a prerequisite, the biocatalytic system must be

Glossary

Bio-based economy: a type of economy that integrates the production of renewable resources with their conversion into bio-based products, bioenergy, food, and feed using existing and novel techniques mainly of industrial biotechnology.
Fast pyrolysis: direct thermochemical decomposition of organic materials in the absence of oxygen. The process enables direct conversion of solid biomass with a low volumetric energy density into a complex liquid product with a high volumetric energy density. The resulting dark brown liquid is commonly named pyrolysis oil, bio-oil, or bio-crude; in this review the term 'pyrolysis oil' is used.
Levoglucosan: monomeric anhydrosugar (1,6-anhydro-β-D-glucopyranose) resulting from the thermal decomposition of cellulose. The most abundant pyrolytic sugar in pyrolysis oil. Biochemical analysis of the levoglucosan metabolic pathways has revealed mechanistic differences between eukaryotic and prokaryotic organisms that are capable of growing on levoglucosan.
Lignocellulosic material: the most abundant renewable organic material. The major building block of plant cell walls and mainly consists of the three polysaccharides cellulose, the polyose hemicellulose, and lignin. The proportion of these constituents depends on the plant species (e.g., hardwood, softwood, or grasses), and varies with age or stage of growth. Generally, lignocellulosic biomass is composed of about 40–50% cellulose, 20–30% hemicellulose, and 10–25% lignin.
Pyrolysis oil: dark brown, acidic, viscous, and energy-rich liquid with a distinctive smoky odor. The complex mixture comprises both polar and non-polar chemicals of different molecular weights that are mainly derived from rapid and simultaneous depolymerization and fragmentation of the three building blocks of lignocellulose: cellulose, hemicellulose, and lignin. The presence of many organic acids within the pyrolysis oil leads to a low pH of 2–3. The large amount of incorporated oxygen (44–60 wt%) is responsible for chemical instability and leads to a wide range of both boiling point temperature and viscosity (35–1000 cP at 40°C). As a

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Box 2. Fast Pyrolysis Conditions and Their Effects on the Composition of Pyrolysis Oil

One of the options to control bio-oil composition is to modify pyrolysis conditions such as the reactor temperature and/or the residence time of vapors and solids. Demirbas investigated the influence of pyrolysis temperature on the yields of compounds in pyrolysis oil obtained from various biomass feedstocks [72]. It was reported that an increase in temperature from 350 to 600°C results in a significant decrease in the concentration of organic acids, but the yields of levoglucosan and furfural also decline with increased pyrolysis temperature. The concentrations of other components such as phenol, methanol, and acetone increase with higher temperatures. Higher temperatures cause the formation of small lignin-derived oligomers, resulting in pyrolysis oil with high viscosity [73]. These results coincide with the degradation profile of lignocellulose during pyrolysis (Figure 1). Hemicellulose decomposes at temperatures between 200 and 300°C, leading to high concentrations of organic acids. If lignocellulose is pyrolyzed at higher temperatures (ca 300–400°C) cellulose also decomposes, forming mostly anhydrosugars such as levoglucosan. Therefore, a pyrolysis temperature of ~350°C results in high concentrations of organic acids and levoglucosan within pyrolysis oil. Because lignin decomposes over a wide temperature range (250–500°C), mainly resulting in monomeric phenolic compounds and pyrolytic lignin, the concentration of lignin-derived compounds increases slowly while the concentration of organic acids and levoglucosan decreases.

Another option is fractionation of the vapors produced during fast pyrolysis. Westerhof and colleagues investigated the feasibility of fractionation during condensation in the pyrolysis unit, thereby steering of the composition of pyrolysis oil by adjusting different reactor and condenser temperatures [6]. They demonstrated that a first condensation of pyrolysis vapors at 70–90°C results in an oily phase containing considerably less water (4–10 wt%) and less organic acids (2–3 wt %); however, this had a high content of sugars (20 wt%) and lignin-derived oligomers (40 wt%) (aromatic compounds). With a subsequent condensation at 20°C, an aqueous condensate is obtained containing low molecular weight organics (40 wt%) such as organic acids (mainly formic and acetic acids), methanol, ethanol, acetol, and hydroxyacetaldehyde. Phase separation can also occur by spontaneous separation of pyrolysis oil during storage, in particular when using high-moisture or ash-rich feedstocks. The resulting two-phase pyrolysis oil consists of a sugar-rich aqueous phase and an aromatic-rich organic fraction.

function of the time and temperature of storage, an increase in viscosity is typically observed, which is caused by reactions with atmospheric oxygen. Typically, pyrolysis oils are single-phase liquids with a density of about 1.1–1.3 kg/m^3. However, high-moisture feedstocks (> 10 wt%) such as annual plants or nonwoody biomass result in phase separation. The resulting two-phase pyrolysis oils consist of a viscous organic fraction and an aqueous fraction.

able to convert this substrate. This is feasible for the low molecular weight molecules of the hydrophilic fraction which contains easily metabolizable components. Among the different organic components, polysaccharide-derived substrates in particular, including anhydrosugars, are potential carbon sources for microbial processes [15], but organic acids mainly including acetic acid and other carbon compounds such as glycolaldehyde and hydroxyacetone can also be potentially suitable microbial substrates [16] (Figure 2). However, many of the identified components have previously been reported to be inhibitory for microbial growth, among them different aromatic compounds and other organic solvents. Furthermore, the above-mentioned properties and highly complex composition may further interfere with existing analytical methods.

To enhance future prospects of utilizing pyrolysis oil via biochemical or microbial conversion, additional knowledge regarding microbial metabolic requirements, bioprocess fundamentals, and pyrolysis process conditions is needed. For complete and efficient utilization of pyrolysis oil fractions, the synergistic combination of interdisciplinary strategies should be addressed in the future. The potential and the challenges of such a strategy are discussed in this article.

Screening and Strain Engineering

Screening or strain engineering for microorganisms is one of the most urgent tasks for efficient biochemical conversion of pyrolysis oil or fractions thereof. In the following section the potential of different components of pyrolysis oil to serve as a substrate for microbial growth is discussed.

Levoglucosan

Several fungi and yeasts as well as a limited number of prokaryotes are able to use anhydrosugars such as levoglucosan (Box 3) that are present in pyrolysis oil. Current studies are mainly focused on the fermentation of levoglucosan to ethanol and lipids [17–19]. Most

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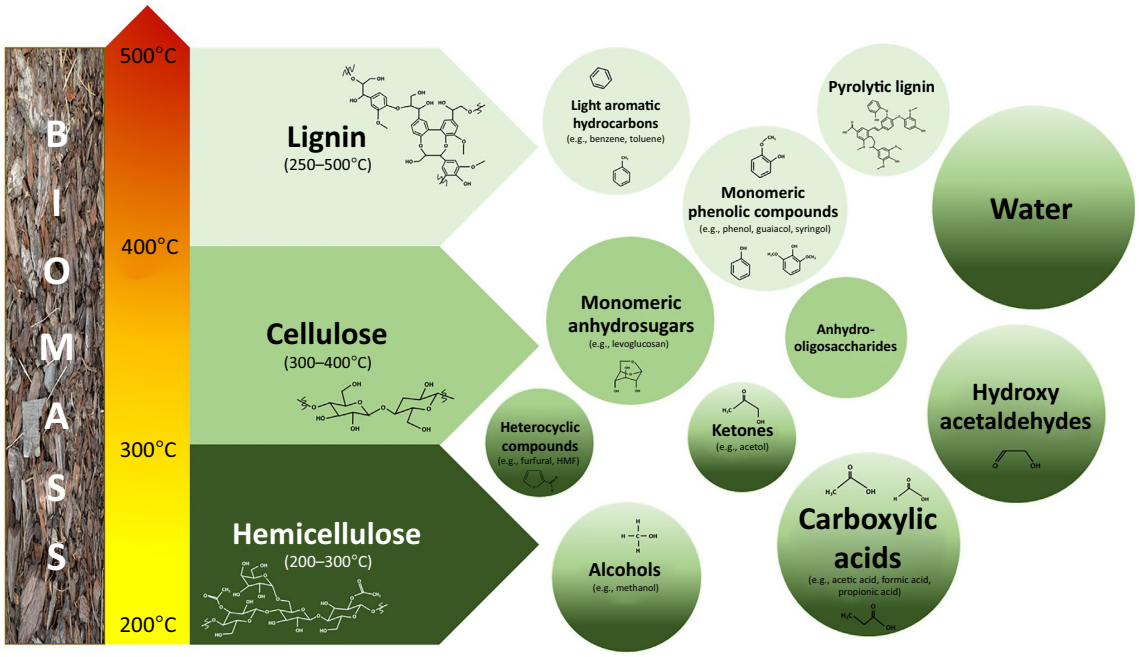

Figure 1. Thermal Decomposition of Lignocellulose. Typical thermal decomposition of the three building blocks of biomass, characteristic temperature range, and the compounds generated by decomposition. The size of circles is proportional to the average fraction amount within pyrolysis oil; the color indicates the source material.

industrial microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae* lack pathways to metabolize levoglucosan. Therefore, current studies are aimed at modifying such strains to enable them to utilize levoglucosan directly. To achieve this the levoglucosan kinase (*lgk*) gene must be functionally expressed (Box 3). This strategy was successfully followed in different studies for the production of ethanol, such as by the expression of LGK from *A. niger* in *S. cerevisiae* H158 [20] or by stable genomic integration of *lgk* from the yeast *Lipomyces starkeyi* YZ-215 in several strains of *E. coli* [18,21]. Furthermore, Linger *et al*. reported levoglucosan metabolism in *P. putida* following a similar approach of *lgk* integration [22]. Recently, Kim *et al*. provided the first example for succinate production from levoglucosan with an engineered *Corynebacterium glutamicum* BL-1 [23]. Therefore, given these studies, establishing levoglucosan metabolism is relatively easy to achieve because only the *lgk* gene needs to be introduced. However, it should be noted that a second approach of establishing levoglucosan metabolism via levoglucosan dehydrogenase has not been investigated in detail, and therefore quantitative data allowing comparison of efficiencies are not yet available (Box 3).

Organic Acids

Organic acids are key components in the metabolism of many microorganisms, and therefore can be utilized as a carbon source by several microorganisms. However, high concentrations of organic acids are often reported to inhibit microbial growth or metabolism [24]. Because pyrolysis oil comprises many organic acids, such as acetic acid (0.5–17 wt%), formic acid (0.3–9.1 wt%), and propionic acid (0.1–2 wt%), it is desirable to improve the tolerance of microorganisms to higher concentrations of these compounds.

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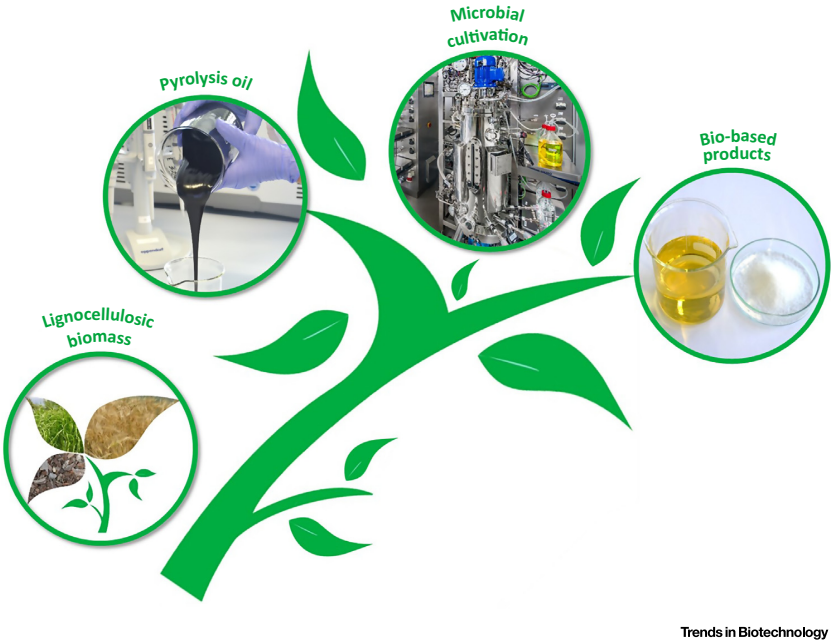

Figure 2. Pyrolysis Oil in the Value Chain of a Bio-Based Economy. The figure shows the envisaged carbon flux from lignocellulose to bioproducts via pyrolysis intermediate steps.

Many bacteria, yeasts, and fungi are able to grow on acetate as a sole carbon source. Acetate metabolism following uptake into the cell is thought to take place via activation to acetyl-CoA by the acetate-activating enzymes acetate kinase and phosphotransacetylase encoded by the *ack* and *pta* genes, respectively. Anaplerotic reactions are a prerequisite for all anabolic reactions originating from the citric acid cycle (TCA cycle). In the case of acetate the key anaplerotic reaction is the glyoxylate cycle [25], and neither pyruvate nor phosphoenolpyruvate (PEP) from glycolysis are available for anaplerotic reactions. Therefore, the genes encoding the two key enzymes of the glyoxylate cycle, isocitrate lyase (*aceA*) and malate synthase (*aceB*), must be present and functionally expressed. One industrially relevant microorganism able to metabolize and grow on acetate as a sole carbon source is *E. coli,* which produces malate synthase and isocitrate lyase. Despite the inhibitory effect of acetate, concentrations of 8 mM (0.5 g/l) in the culture medium have been reported to sustain 50% of the typical maximum growth rate [26]. Chong *et al*. engineered *E. coli* strains with enhanced sodium acetate tolerance up to concentrations of 250 mM (15 g/l) by introducing random mutations into one of the central metabolic regulators, the *crp* gene [27]. The generation of succinate from acetate as sole carbon source by an engineered *E. coli* has been reported by Li *et al*. [28]. The authors investigated strategies of metabolic engineering by downregulating the TCA cycle, redirecting gluconeogenesis, or enhancing the glyoxylate pathway. By contrast, the industrially employed *B. subtilis* does not possess the key enzymes of the glyoxylate cycle and is unable to use acetate as a sole carbon source. Hence, Kabisch *et al*. transferred the operon encoding isocitrate lyase (*aceB*) and malate synthase (*aceA*) from *B. licheniformis* into *B. subtilis*, resulting in a modified *B. subtilis* strain able to grow on acetate [29]. This is a comparatively simple method to establish heterologous acetate metabolism. Like *E. coli*, the yeast *S. cerevisiae* possesses the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, enabling *S. cerevisiae* to metabolize acetate.

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Box 3. Levoglucosan – A Potential Carbon Source for Microbial Conversion

Levoglucosan is among the most promising constituents of pyrolysis oil for microbial conversion. Because of its relatively high concentrations, levoglucosan has been targeted as potential carbon source in the past [18,22,74]. Levoglucosan can be converted into glucose by acidic hydrolysis [75] and subsequently used as a fermentative substrate [76,77]. However, the use of acids produces heat and forms additional inhibitors. Therefore, it is desirable to employ biocatalysts that can directly convert pyrolysis oil into biorenewable chemicals with minimal processing steps. Even though levoglucosan is not commonly present in nature, it can be found where forest fires or other types of biomass burning incidents have occurred [77]. In addition to a variety of yeast and fungi strains, some bacterial species are also able to utilize levoglucosan directly as a carbon and energy source for growth [74,75,77–79]. The metabolism of levoglucosan in eukaryotes (Figure IA) is initiated by direct phosphorylation to glucose-6-phosphate by levoglucosan kinase (LGK) [75].

In contrast to the pathway in eukaryotic organisms, the metabolic pathway of levoglucosan in prokaryotes is not fully understood. It has been suggested that prokaryotic microorganisms metabolize levoglucosan through at least three steps and require NAD^+ as a cofactor [78]. For a bacterium belonging to the genus *Arthrobacter* it could be shown that a levoglucosan dehydrogenase catalyzes the dehydrogenation of levoglucosan to 3-keto levoglucosan, then to 3-keto glucose, and finally to D-glucose by using NAD^+ as an electron acceptor (Figure IB) [79].

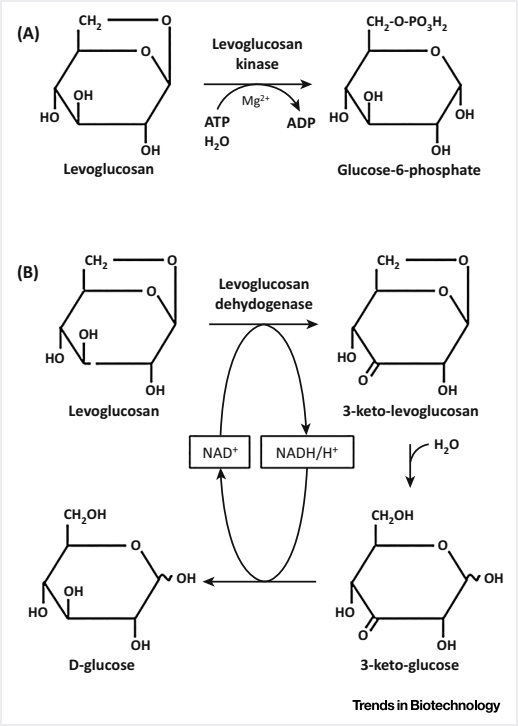

Figure I. Enzymatic Conversion of Levoglucosan to Microbially Utilizable Substrates. Levoglucosan can be converted in a single step to glucose-6-phosphate (A) or converted in three steps to D-glucose (B).

Acid stress and tolerance mechanisms in eukaryotes are often studied in *S. cerevisiae*. For advanced tolerance engineering, two mechanisms of acid stress response may be exploited, namely the initial acid shock response as the first step of adaptation to acidic conditions, and acid adaptation itself, that maintains integrity under acidic conditions. The transcription activators Aft1 and Haa1 are reported to be key acid stress regulators [30–33] and Haa1-regulated genes have been shown to be upregulated under acetate stress conditions (0.6%) [34].

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Interestingly, acetate as a co-substrate of glucose was beneficial for citric acid production in the mold *A. niger* [35,36].

In addition to acetate, formate is also a potential microbial feedstock. One metabolic pathway by which microbes can grow on formate as the sole carbon source is the oxidation of formate to CO_2 by formate dehydrogenase, and the reducing power generated supports carbon fixation and provides the cell with energy or condensed with another metabolic intermediate. Bar-Even extensively discussed the natural enzymes and pathways involved in formate assimilation and compared them with proposed synthetic counterparts [37]. Propionate is metabolized via the methylcitrate cycle (MCC), which is present in various bacteria and yeasts. However, as in bacteria, no comprehensive studies on the metabolism of propionic acid as a carbon source have been pursued for yeasts or molds. Dang *et al*. investigated *S. cerevisiae* QH01 for its ability to grow on small organic acids, and reported growth on formic acid, acetic acid, and propionic acid [38].

Because acetic acid is the main organic acid within pyrolysis oil, an efficient acetate-converting microorganism may provide a solid basis for biotechnological utilization of this substrate.

Monohydric Alcohols

No monohydric alcohols other than methanol have been reported to be present at significant concentrations in pyrolysis oils. The ability to assimilate methanol is mainly attributed to oxidation of methanol to formaldehyde, followed either by oxidation to CO_2 or by assimilation into central carbon metabolism.

For the utilization of methanol Koopman *et al*. introduced the *hps* and *phi* genes from *Bacillus brevis* into *P. putida* S12 [39]. These genes encode two key steps of the ribulose monophosphate pathway (RuMP). Cultivation of engineered *P. putida* S12 in chemostats fed with methanol or formaldehyde as a co-substrate resulted in increased biomass concentrations of *P. putida* S12. Other common industrial microbial platform organisms such as *E. coli* and *C. glutamicum* are not normally able to utilize methanol as a sole carbon and energy source. In a similar approach Müller *et al*. heterologously expressed the *hps* and *phi* genes from *Mycobacterium gastri* as a fusion protein in *E. coli* [40]. With the aim of using methanol as an auxiliary carbon source in a sugar-based medium for microbial production processes, Witthoff *et al*. engineered *C. glutamicum* ATCC 13032 to carry out methanol oxidation and formaldehyde assimilation via the RuMP [41]. To increase the methanol tolerance of *C. glutamicum*, Lessmeier and Wendisch performed genome sequence analysis of an evolutionarily methanol-adapted Leßmeier*C. glutamicum* strain, and reported that two single-nucleotide polymorphisms (SNPs) significantly increase the tolerance of *C. glutamicum* to methanol [42].

Methanol is mostly used as a co-substrate rather than a primary source of carbon to increase final biomass yields. Owing to its low abundance in pyrolysis oil, methanol can be regarded as a secondary target for strain engineering.

Further Compounds

During pyrolysis, numerous compounds are generated at low concentrations, and are either still unidentified and/or are considered to be inhibitory to microbial growth [5]. These inhibitory properties are mainly attributed to aromatic compounds (e.g., phenol, guaiacol, syringol), heterocyclic compounds (e.g., furfural), and other organic solvents (e.g., acids, alcohols). Pyrolytic lignin, the water-insoluble fraction of pyrolysis oil, in particular contains many phenolic compounds [43] which can be inhibitory to microorganisms above a certain concentration. The global mechanisms of resistance to phenolic compounds in *S. cerevisiae* or other microorganisms have not yet been completely elucidated. This is mainly due to heterogeneity and lack of accurate analytical methods.

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However, many microorganisms are able to tolerate or even degrade 'toxic' components in pyrolysis oil. This is exemplified by *P. putida* KT2440 which has been known for decades to be able to assimilate toluene. In addition to *P. putida*, *C. glutamicum* can also use a wide variety of aromatic compounds including phenol, *p*-cresol, and benzoate. Lee *et al*. reported that *C. glutamicum* ATCC 13032 can completely degrade 8.5 mM (0.8 g/l) phenol [44]. The authors also reported that the production of glutamate and proline is enhanced by the presence of phenol in the culture medium. Hasan and Jabeen isolated a *Pseudomonas* sp. and a *B. subtilis* strain with high tolerance to phenol [45]. Tebbouche *et al*. reported that *A. niger* LSTE-AH1 is able to completely degrade initial phenol concentrations of 200 mg/l [46]. An obvious strategy for enhanced resistance to phenolic inhibitors in lignocellulose hydrolysates is the heterologous expression of laccase from white rot fungus. Such an approach has been successfully established by Larsson *et al*. [47].

Among the inhibitors formed during pyrolysis, the furanic aldehydes are considered to be the compounds of most concern. Tolerance to furfural is therefore one of the main targets in engineering the utilization of pyrolysis oil. Only a few microorganisms, mainly Gram-negative bacteria, have been reported to utilize furfural as a sole carbon source [48]. Lee *et al*. isolated five bacteria from wastewater treatment facilities which are able to use furfural as a sole carbon source, and a concentration of 17 mM (1.6 g/l) of furfural reduced the growth rate of a *P. putida* isolate by 50% [49]. Furthermore, *B. subtilis* DS3 was reported to utilize furfural as a sole carbon source [50]. It is described that this strain is able to degrade 31.2% furfural and tolerates furfural concentrations of up to 6 g/l. Furthermore, Tsuge *et al*. described the detoxification of furfural by *C. glutamicum* ATCC13032 under both aerobic and anaerobic conditions [51].

Despite the assumed toxicity of pyrolysis oils, *P. putida* is able to utilize and tolerate a wide variety of these compounds. The search for suitable microorganisms to establish pyrolysis oil as a platform substrate for industrial biotechnology is mainly restricted to major compounds such as monomeric phenols and aromatics. However, for maximized productivity, fed-batch or continuous processes are typically chosen in industrial biotechnology. It is therefore crucial to consider the entire spectrum of pyrolysis oil constituents for successful process design because even inhibitory substances at low concentrations may ultimately accumulate in the culture broth during the feeding procedure. Some microorganisms such as *P. putida* and *B. subtilis* may successfully be used to degrade some of these inhibitory substances, thereby preventing the accumulation of inhibitors in the medium. Furthermore, addressing unspecific or specific mechanisms of microbial tolerance towards inhibitory compounds in pyrolysis oil is a promising new field of study. Work to date suggests that engineering microbial tolerance to toxic compounds has great potential, although many of the mechanisms are not yet understood in detail.

Pretreatment and Bioprocessing: Converting Pyrolysis Oil into Microbially Utilizable Feedstocks

To reduce the overall inhibitory effects of pyrolysis oil, adaptation of pyrolysis conditions (Box 2), fractionation of pyrolysis oil for enrichment of desirable substrates, and detoxification of pyrolysis oil before cultivation are viable options. Various studies on fast pyrolysis have been performed to achieve maximum liquid yields of pyrolysis oil. However, the process conditions for maximizing liquid yields usually do not address the quality and composition of pyrolysis oil.

Wang *et al*. investigated the biotechnological use of the aqueous phase of rice husk-derived pyrolysis oil as a carbon source, and suggested that it can be used by recombinant *E. coli* MG-PYC for cell growth and succinic acid production [52]. The potential of an aqueous phase obtained from fractional pyrolysis of wood from waste pallets as a carbon source for microbial fermentation was investigated by Lian *et al*. [53]. The authors examined *Cryptococcus curvatus*

Trends in Biotechnology

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ATCC 20509 for its ability to utilize carboxylic acids present in an aqueous phase for the production of lipids. After different purifications steps such as neutralization, evaporation of acetol and hydroxyacetaldehyde, dilution, and detoxification with activated carbon, the final concentration of acetate was 20 g/l. Using this as a feedstock enabled the production of 6.9 g/l dry biomass and 2.2 g/l lipids, which is remarkably high in comparison to other processes. Overall microbial biomass yields were higher when employing the pyrolytic aqueous fraction compared to pure acetate, and this may be attributed to the presence of additional carbon sources such as formate within pyrolysis oil. In a similar study, Gong *et al*. achieved a lipid concentration of 8.1 g/l from 15.9 g/l of acetate [54].

Post-pyrolysis detoxifying procedures of the aqueous phase mainly include over-liming [5,55], successive adsorption on activated carbon to remove remaining phenols [17,56], air stripping [57], and solvent extraction for the removal of the phenol-rich organic phase [17,58].

In addition to lignocellulosic biomass including forest and agricultural residues, plastic or tire wastes are also potentially suitable materials for pyrolysis. Depending on the chosen raw material, the composition of pyrolysis oil varies greatly. Moita and Lemos reported the use of the entire lignocellulosic pyrolysis oil as a possible microbial feedstock for the production of short-chain polyhydroxyalkanoates (PHAs) by mixed microbial cultures [59]. The pyrolysis oil used for this study was obtained by fast pyrolysis of residues from poultry farming (chicken beds) to provide a carbon source for selection of a mixed microbial culture able to produce PHAs under saturation conditions. PHAs are produced under nutrient limitation with carbon excess [60] and are stored intracellularly as vesicles. Pyrolysis oil is a poor nitrogen source but is rich in carbon, suggesting that it may have potential as an effective feedstock for the production of PHAs. Moita and Lemos reported maximum PHA yields of 0.092 g/g cell dry weight of a co-polymer composed of 70% hydroxybutyrate (HB) and 30% hydroxyvalerate (HV) monomers achieved by cultivation of mixed cultures containing dilute pyrolysis oil (1:176 pyrolysis oil to mineral medium) as a carbon source [59]. In a further study it was claimed that there is no need to detoxify pyrolysis oil to use it as carbon source for the mixed cultures employed [16].

In comparison to carbon sources generally used in industrial biotechnology, such as molasses, starch, glucose, or food-processing byproducts, pyrolysis oils have an extraordinarily complex and demanding composition. There are three main challenges for the biotechnological conversion of this carbon source: (i) general toxicity, (ii) an overall complex composition, and (iii) a low concentration of microbially accessible molecules.

Common cultivation methods such as submerged homogeneous cultures do not seem to be an adequate strategy for using pyrolysis oil as a substrate. This is because inhibitory or toxic compounds will ultimately accumulate in fed-batch cultivations which are typically favored due to higher productivities. Therefore, there is a demand to develop new strategies which will allow the utilization of easily accessible constituents of pyrolysis oil while simultaneously preventing the buildup of inhibitory components.

An interesting example that illustrates a new research perspective for the biotechnological utilization of pyrolysis oil is the production of the bioplastic PHA by *Cupriavidus basilensis*. Wierckx *et al*. isolated the Gram-negative bacterial strain *C. basilensis* HMF14 from enrichment cultures with 5-hydroxy-2-methylfurfural (HMF) as the sole carbon source [61]. This isolate was able to grow on inhibitory constituents including HMF, furfural, and aromatic compounds derived from wheat straw hydrolysis. Interestingly, no growth was observed on sugars including xylose, arabinose, or mannose, and not even on glucose. Therefore, *C. basilensis* HMF14 could be used to remove inhibitors while producing PHAs, preserving the sugars for subsequent cultivations. Successful cultivations were reported on 3.5 mM HMF, furfural, furfuryl alcohol, or

Trends in Biotechnology

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furoic acid as the sole carbon source, and a maximum optical density of ~1.0 was achieved. *C. basilensis* HMF14 was also able to grow in the presence of up to 15 mM HMF or furfural; however, concentrations above 6 mM had an inhibitory effect on the cells, resulting in decreased biomass. Another interesting study showed that *C. necator* JMP134 is able to utilize lignin monomers as a carbon source to produce polyhydroxybutyrate (PHB) [62].

Flow-through bioreactors provide an obvious technical solution to the problems described because they prohibit the accumulation of inhibitory substances. For operation in flow-through mode, biofilm reactors and compartment bioreactors are the set-up of choice. These reactors can potentially consume the limiting substrate while simultaneously avoiding the buildup of inhibitory substances. In both reactor types, biomass accumulates inside the reactor. Therefore, intracellular products have a natural advantage over extracellularly secreted products. The formation of biofilms on surfaces is a universal bacterial strategy for survival and for optimum positioning with regard to available nutrients, which is beneficial in view of the inhibitory effect of pyrolysis oil. As one example, Khiyami and coworkers reported the degradation of corn stover and corn starch pyrolysis liquors using a biofilm reactor design employing a culture of immobilized *P. putida* [63].

Compartment bioreactors allow different bacteria to be confined to individual reaction spaces separated by membranes. As in biofilm reactors, this arrangement facilitates the flow-through mode and intracellular products are advantageous. Both technological approaches may enable the utilization of pyrolysis oil as carbon source for biotechnological conversion. Further research topics include the basics of biochemical kinetics, layout, and process design, as well as the mode of harvesting.

Overall, it is clear that strategic and cooperative use of strains and suitable technologies will be necessary for efficient and complete degradation of pyrolysis oil.

Concluding Remarks and Future Perspectives

Synergistic optimization of chemical and biochemical conversion processes may facilitate the economics of the pyrolysis process as a whole. The utilization of pyrolysis oil remains a challenge for a microbial bio-based economy (see Outstanding Questions). This is because of its highly complex and variable composition, as well as its adverse effects on microbial growth and resistance to analytical procedures. The identification of appropriate microorganisms such as *C. basilensis* and successful tolerance engineering in yeasts are examples of current research topics. In addition, pretreatment and process engineering-based approaches will be crucial for the utilization of pyrolysis oil as an alternative substrate for a bio-based economy.

Outstanding Questions

Can a bioprocess be designed to circumvent the restrictions resulting from the chemical nature of pyrolysis oil? Its relatively low content of bio-accessible carbon in combination with inhibitory properties make the use of common bioprocess designs unfavorable. Therefore, new reactor designs such as biofilm reactors or membrane reactors may help to overcome these challenges.

How can different research fields be integrated to achieve synergistic process development for pyrolysis fractionation and bioconversion as well as chemical conversion? An efficient process should take into account the complete value chain, including total utilization of all available carbon.

How can we address tolerance engineering given that there are multiple targets and the lack of information regarding the mechanism of inhibition? Methods must be developed to analyze microbial resistance mechanisms and engineer their adaptation to permit further application in synthetic biology.

What are the key parameters of life-cycle assessments to be evaluated in future research? The main supply of lignocellulosic materials is in remote and/or rural regions, thus calling for a decentralized distribution of bio-based manufacturing sites. Life-cycle assessments should include all aspects of production of lignocellulose, transport, depolymerization, and chemical and biotechnological conversion, as well as integration into existing value chains.

How can the pyrolysis process be modified to produce fractions that are most suitable for fermentation?

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1.5 Research Proposal

This research project is part of the Bioeconomy Research Program Baden-Württemberg – an interdisciplinary program consisting of three research networks: biogas, lignocellulose and microalgae. This thesis is attributed to the research area lignocellulose, which focuses on the development and improvement of production, conversion and valorization of lignocellulosic biomass. Projects within this research area are highly networked, whereby this work mainly cooperates with researchers from the Karlsruhe Institute of Technology (KIT), Institute of Catalysis Research and Technology (IKFT), who are involved in the Karlsruhe bioliq® process.

The main goal of the bioliq® process is the production of tailored synthetic fuels based on herbaceous, ash-rich lignocellulosic biomass residues like wheat straw (Pfitzer *et al.* 2016, Dahmen *et al.* 2017). The concept combines a decentralized production of an energy-dense intermediate product by fast pyrolysis of biomass in a twin-screw mixing reactor with its central conversion into syngas by gasification, which in turn is a versatile intermediate for production of various base chemicals or synthetic fuels. The applied two-stage condensation allows a controlled formation of two condensates: a viscous organic condensate (OC) rich in C6 compounds (sugars and aromatic compounds) with < 20 % water obtained at condensation temperatures between 60 and 90 °C and an aqueous condensate (AC) consisting of up to 85 % water and C1 - C4 molecules (e.g., carboxylic acids, methanol, ethanol, acetol, hydroxyacetaldehyde) formed at around 30 °C (Pfitzer *et al.* 2016). The monomeric substances within the two condensates OC and AC were analyzed by Thünen Institute Hamburg by GC-MS and are listed in the appendix (Table S1). While the organic fraction can be further refined to produce transportation fuels (Dahmen *et al.* 2012, 2017), there are only a few studies on utilization of the aqueous fraction so far (Lian *et al.* 2012, Liang *et al.* 2013). As a future bioeconomy relies on the efficient and sustainable utilization of bio-based resources, it is important that for all product and side streams, which accrue during biorefining of biomass, adequate applications are found. Since fast pyrolysis bio-oil and its fractions comprise microbially metabolizable components such as sugars and organic acids, their utilization as carbon sources in biotechnological processes is a promising approach.

In order to the development of a future bio-based economy, the intention of this thesis is to evaluate the potential and challenges of fast pyrolysis bio-oil derived

from lignocellulosic biomass as an alternative and sustainable carbon source for bacterial bioconversion. For this purpose the work is divided into three parts corresponding to the three publications established during this work and addressing the following aspects:

Part I:

- Investigation of different pretreatment strategies of bio-oil fractions to overcome their strong inhibitory effects and unsuitability for common analytical methods
- Cultivation of *Pseudomonas putida* KT2440 as a reference system on these different pretreated bio-oil fractions
- Evaluation of different pretreatment strategies for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass

Part II:

- Evaluation of the main small organic acids present in bio-oil with respect to their suitability as feedstocks for bacterial growth
- Cultivation of four biotechnological production hosts *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis* and *Corynebacterium glutamicum* on different concentrations of single and mixtures of small organic acids
- Investigation of bioconversion of pretreated bio-oil fractions with respect to the suitability of the selected bacterial strains to tolerate inhibitory substances within lignocellulosic-based feedstocks

Part III:

- Investigation of growth behavior of a genetically engineered *Pseudomonas putida* KT2440 strain and its simultaneous heterologous production of rhamnolipid biosurfactants on bio-oil derived small organic acids and pretreated fractions
- Implementation of a novel value-added process chain using bio-oil derived small organic acids and fractions to produce rhamnolipid biosurfactants as exemplary value-added product

Chapter

2

Publications

I Pretreatment strategies for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass

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ORIGINAL RESEARCH

WILEY GLOBAL CHANGE BIOLOGY BIOENERGY

Pretreatment strategies for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass

Stefanie Arnold[1] | **Karin Moss**[1] | **Nicolaus Dahmen**[2] | **Marius Henkel**[1] | **Rudolf Hausmann**[1]

[1]Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150k), University of Hohenheim, Stuttgart, Germany

[2]Karlsruhe Institute of Technology (KIT), Institute for Catalysis Research and Technology, Karlsruhe, Germany

Correspondence
Marius Henkel, Institute of Food Science and Biotechnology, Department of Bioprocess Engineering (150k), University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany.
Email: marius.henkel@uni-hohenheim.de

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Abstract

This work evaluates a biorefinery approach for microbial valorization of bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass. Different methods are presented for the pretreatment of the low-sugar complex bio-oil consisting of organic condensate (OC) and aqueous condensate (AC) to overcome their strong inhibitory effects and unsuitability for common analytical methods. Growth of *Pseudomonas putida* KT2440, which was chosen as a reference system, on untreated bio-oil fractions was only detectable using solid medium with OC as sole carbon source. Utilization of a pretreated OC which was filtered, autoclaved, neutralized and centrifuged enabled growth in liquid medium with significant remaining optical instability. By subjecting the pretreated fractions to solid phase extraction, more stable and less inhibitory bio-oil fractions could be obtained enabling the appliance of common analytical methods. Furthermore, this pretreatment facilitated growth of the applied reference organism *Pseudomonas putida* KT2440. As there is currently no convincing strategy for reliable application of bio-oil as a sole source of carbon in industrial biotechnology, the presented work depicts a first step toward establishing bio-oil as a future sustainable feedstock for a bio-based economy.

KEYWORDS

bioeconomy, bio-oil, lignocellulosic biomass, pretreatment, *Pseudomonas putida* KT2440, pyrolysis

1 | INTRODUCTION

The most abundant nonfood renewable raw material is lignocellulosic biomass, which may present a sustainable alternative platform to petrochemicals (Anwar, Gulfraz, & Irshad, 2014; Iqbal, Kyazze, & Keshavarz, 2013; Menon & Rao, 2012). A significant challenge, however, lies in its conversion because lignocellulose is a plant-based, sturdy and compact biocomposite material consisting of lignin,

Abbreviations: AC, aqueous condensate; AC_{FANC}, aqueous condensate after filtration, autoclaving, neutralization and centrifugation; AC_{SPE}, aqueous condensate after solid phase extraction; OC, organic condensate; OC_{FANC}, organic condensate after filtration, autoclaving, neutralization and centrifugation; OC_{SPE}, organic condensate after solid phase extraction; SPE, solid phase extraction.

cellulose and hemicellulose. In the last decades, a number of conversion methods have been developed mainly focusing on the production of second-generation biofuels (Brethauer & Studer, 2015; Dahmen et al., 2016; Maurya, Singla, & Negi, 2015; Seidl & Goulart, 2016). One of such a conversion method is fast pyrolysis—a thermochemical method which converts lignocellulosic biomass in the absence of oxygen mainly into an energy-rich liquid referred to as bio-oil, also referred as to bio-oil (Bridgwater, Meier, & Radlein, 1999).

One example for a fast pyrolysis process with fractional condensation, which addresses pyrolysis of nonwoody biomass such as herbaceous, ash-rich biomass like wheat straw, was shown by Pfitzer et al. (2016) (bioliq® process, Karlsruhe Institute of Technology (KIT), Germany). The applied two-stage condensation allows a controlled formation of two condensates: a viscous condensate rich in organic substances with <20 wt% water (OC) obtained at condensation temperatures between 60 and 90°C and an aqueous condensate (AC) consisting of up to 85 wt% water and water-soluble organic compounds formed at around 30°C (Figure 1; Pfitzer et al., 2016). An excerpt of their composition is shown in Table 1. These condensates are mixed with pyrolysis char in different proportions forming energy-rich and free-flowing bioslurries, which are further used for gasification to generate synthetic fuels (Dahmen, Henrich, Dinjus, & Weirich, 2012). Depending on the applied biomass and the mixing ratio, unexploited side streams can accrue. As a future bioeconomy relies on the efficient and sustainable utilization of renewable resources, it is important that for all products and side streams, which accrue during an overall conversion process of lignocellulosic biomass an adequate valorization is found. Furthermore, it should be taken into account that the use of biomass as a feedstock for energy and fuel production is restricted by its relatively low volumetric energy content, seasonality, and discrete geographic availability (FitzPatrick, Champagne, Cunningham, & Whitney, 2010; Lipinsky, 1981).

An alternative application of lignocellulosic-based bio-oil and its fractions is its use as a carbon source for microbial cultivation (Arnold, Moss, Henkel, & Hausmann, 2017; Islam, Zhisheng, Hassan, Dongdong, & Hongxun, 2015; Jarboe, Wen, Choi, & Brown, 2011). Bio-oil is mainly composed of water, pyrolytic lignin, and a wide variety of organic components such as organic acids, sugars, alcohols, aldehydes, ketones, and phenolic components (Piskorz, Scott, & Radlein, 1988). Especially, pyrolytic sugars and organic acids are of particular interest to be used as carbon sources by microorganisms (Bennett, Helle, & Duff, 2009; Chi et al., 2013; Kim, Um, Bott, & Woo, 2015; Layton, Ajjarapu, Choi, & Jarboe, 2011; Lian et al., 2010; Lian, Garcia-Perez, & Chen, 2013; Lian, Garcia-Perez, Coates, Wu, & Chen, 2012; Linger, Hobdey, Franden, Fulk, & Beckham, 2016; Prosen, Radlein, Piskorz, Scott, & Legge, 1993). But bio-oil also comprises many unidentified substances, as well as components which are inhibitory to microbial growth such as furans, phenolic compounds, and ketones (Chi et al., 2013; Jarboe et al., 2011; Lian et al., 2010; Prosen et al., 1993). Furthermore, due to the rapid quenching in pyrolysis process, bio-oil is not at equilibrium and changes chemically and physically with time if no measures for stabilization are taken (Diebold, 2000). Especially, the presence of reactive aldehydes and ketones, organic acids, but also the presence of char cause reactions within various bio-oil functionalities and contribute toward the instability of bio-oil.

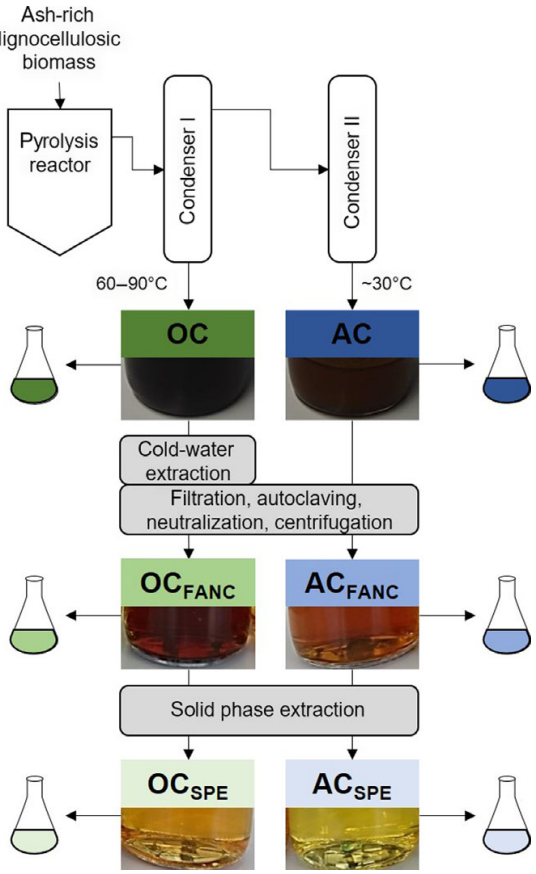

FIGURE 1 Schematic diagram of the different pretreatment steps and its products originating from condensable vapor of fast pyrolysis process with a two-stage condensation. The resulting organic condensate (OC) and aqueous condensate (AC) are furthermore subjected to filtration, autoclaving, neutralization, and centrifugation (FANC). Subsequently, OC_{FANC} and AC_{FANC} are subjected to solid phase extraction (SPE)

TABLE 1 Composition of the two bio-oil phases determined by GC-MS and typical ranges extracted from the literature (Islam et al., 2015)

Compound (wt%)	OC	AC	Typical range
Formic acid	n.a.	n.a.	0.3–9.1
Acetic acid	5.004	4.492	0.5–17.0
Propionic acid	1.302	0.404	0.1–2.0
Methanol	n.d.	1.689	0.4–8.2
Ethylene glycol	1.258	0.437	0.7–2.0
Levoglucosan	0.965	n.d.	0.1–30.5
Acetol	4.631	3.484	0.2–7.4
1-Hydroxy-2-butanone	0.844	0.49	0.3–1.3
2-Cyclopenten-1-one	0.308	0.262	0.3–1.5
Furfural	0.265	0.281	1.5–3.0
Phenol	0.384	0.041	0.1–3.8
Cresol (o-,p-,m-)	0.455	0.058	1.0–2.5
Guaiacol	0.469	0.104	2.8–2.8
Syringol	0.556	0.011	0.7–4.8
Isoeugenol	0.524	n.d.	0.1–7.2

Note. AC: aqueous condensate; OC: organic condensate; n.a.: data not available, n.d.: not detectable.

Different strategies have been developed to prevent inhibition including bio-oil fractionation (Pollard, Rover, & Brown, 2012; Westerhof et al., 2011), detoxification (Chi et al., 2013; Li et al., 2013; Lian et al., 2010, 2012 ; Prosen et al., 1993; Vitasari, Meindersma, & Haan, 2012), and improvement of the tolerance of biocatalysts (Chan & Duff, 2010; Liang et al., 2013; Wang et al., 2012; Yu & Zhang, 2003).

Regarding bio-oil fractions produced from wheat straw by fast pyrolysis in the bioliq® plant at Karlsruhe Institute of Technology (KIT, Karlsruhe, Germany), two studies have already been reported investigating their potential as carbon source for microbial cultivation (Dörsam et al., 2016; Lange et al., 2017). Noticeable is that the sugar content of these fractions is very low in the OC fraction or even nonexistent in AC. The major component present in these fractions is acetic acid (Table 1), which can be a potential carbon source for some microorganisms (Berg, Tymoczko, & Stryer, 2002; Gerstmeir et al., 2003; Li et al., 2016). In 2016, Dörsam et al. determined the bio-oil tolerance limits of fungi, as well as investigated the reason for their low tolerance level by assessing 11 substances from bio-oil in different concentrations regarding the production of malic acid. The authors used the organic-rich bio-oil fraction of the so-called organic condensate (OC). For tolerance experiments, cultivations were performed on solid medium. Among the 22 screened fungi, 15 showed tolerance toward OC in minimal medium agar plates while growing on glucose. Cultivations with OC as sole carbon source suggest that only *Aspergillus oryzae* is able to grow on up to 1% of OC; however, quantitative data are not provided. Beside OC also an aqueous bio-oil phase is formed during the two-stage condensation in the KIT fast pyrolysis plant. In 2017, Lange et al. evaluated the suitability of *Corynebacterium glutamicum* to grow on this so-called aqueous condensate (AC). The authors reported that after supplementation of minimal medium with AC, turbidity analyses were no longer possible. Therefore, they established a protocol for pretreatment of AC consisting of four steps: pH adjustment, centrifugation, filtration, and heat treatment, aiming for a fermentable substrate by removing residual oils and solids as well as diminishing volatile substances such as methanol. However, also with the addition of pretreated AC to the medium growth was not detectable. Only supplementation of yeast extract to pretreated AC enabled growth of an engineered *Corynebacterium glutamicum* and the production of 1,2-propanediol.

Even though the bioconversion of bio-oil was investigated in the past, studies are mainly based on cultivations with sugar-rich, hydrolyzed, and detoxified bio-oil fractions added to complex media, which mostly include yeast extract and peptone (Bennett et al., 2009; Chan & Duff, 2010; Lian et al., 2010; Luque et al., 2014; Prosen et al., 1993; Sukhbaatar et al., 2014; Wang et al., 2012; Yang et al., 2011; Yu & Zhang, 2003). Furthermore, their focus generally lies on product formation and less on the biomass generation. As of today, the effect of reactivity of bio-oil during cultivation by quantitative means such as suitable investigation of supplemented culture medium over time as negative control has not been reported in detail.

In this article, the challenges of using sugar-poor bio-oil fractions as sole carbon sources for bacterial cultivation are described. Different pretreatment methods are presented to address problems such as reactive and inhibitory substances in bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass. Using the example of *Pseudomonas putida* KT2440, which is generally regarded as an organic solvent tolerant bacterial strain capable of degrading aromatic hydrocarbons and using a wide range of carbon sources (Cruden, Wolfram, Rogers, & Gibson, 1992; Inoue & Horikoshi, 1989; Ramos, Duque, Huertas, & Haïdour, 1995; Weber, Ooijkaas, Schemen, Hartmans, & Bont, 1993), a potential biorefinery route for microbial valorization of bio-oil is shown.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals used in this study were either purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) if not stated otherwise.

2.2 | Bio-oil fractions and their pretreatments

The two bio-oil fractions organic condensate (OC) and aqueous condensate (AC) used in this study were prepared from wheat straw by fast pyrolysis in the bioliq® plant at KIT in Karlsruhe, Germany (Pfitzer et al., 2016). The substances within the two condensates were analyzed by Thünen Institute Hamburg by GC-MS. As a result of unspecific pyrolytic reactions, OC and AC contain hundreds of different molecules, many of which have not been identified. This is most prominent in OC, where pyrolytic lignin and unidentified components constitute a major part of dry weight. An excerpt is shown in Table 1 comprising the most abundant substances in either OC or AC (above 0.1 wt%) and compared with typical ranges in the literature (Islam et al., 2015). OC is low in sugar (in sum 1.63 wt% and thereof 0.965 wt% levoglucosan), and AC even contains no monomeric sugars. Solids in OC are usually <10 wt% and the water content is adjusted between 12 and 18 wt% by varying the condensation temperature (Pfitzer et al., 2016). AC consists of up to 85 wt% water including water-soluble organic compounds and practically no solids. Its pH is ~2.5. To maintain integrity to favor stability, OC samples were stored at 4°C and AC at −21°C until further use. To obtain stable and fermentable bio-oil fractions, various pretreatment methods were applied. One of the first purification steps of OC was an extraction. The aim was to extract water-soluble substances such as organic acids and sugars from bio-oil and get an aqueous extract rich in these substances. For this, OC was slowly added to ice-cooled water at the ratio of 1:6 while mixing with a stirrer. A filtration with a bottle top filter with PES membrane (pore size: 0.22 µm; membrane Ø: 75 mm; volume: 500 ml) obtained from Carl Roth GmbH (Karlsruhe, Germany) was performed for both OC extract and AC to remove pyrolytic lignin (water-insoluble fraction) and other solid substances. This modified approach is described by Scholze and Meier (2001), who used this procedure for the characterization of pyrolytic lignin. The resulting filtrates were autoclaved, neutralized, and centrifuged at 4,700 rpm for 15 min, whereby the middle phase represents the final aqueous fractions OC_{FANC}, respectively, AC_{FANC} (Figure 1), which were sterile-filtered at the end. Neutral and aromatic compounds within OC_{FANC} and AC_{FANC} were removed using solid phase extraction (SPE) cartridges (Strata-X 33 µm Polymeric Reversed Phase, 200 mg stationary phase, specific surface of 800 m^2/g, Phenomenex Ltd., Aschaffenburg, Germany). Extraction of each sample was performed with a fresh column, by employing 20 ml of OC_{FANC} and $AC_{FANC,}$ which were passed through the column in a single run according to the manufacturers' recommendations. The neutrals and aromatics bind at the column and the sterile-filtered flow-through was used as a more purified fraction called OC_{SPE}, respectively, AC_{SPE} (Figure 1). All pretreated bio-oil fractions were stored at 4°C.

2.3 | Microorganism, culture medium, and conditions

The used bacteria strain *P. putida* KT2440 (DSM 6125) was obtained from the DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *P. putida* was grown at 30°C and 120 rpm. For the preculture LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) was used. After ~18 hr of incubation, the cells were centrifuged (4,700 rpm, 4 min, 20°C) and the cell pellet was washed with 1:1 (v/v) 9 g/L NaCl solution, centrifuged again and resuspended in fresh 9 g/L NaCl solution. The main medium was inoculated with OD_{600} 0.1. The main medium was adapted to Wilm's KP_i medium (Wilms et al., 2001): 6.58 g/L K_2HPO_4, 1.64 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 2 g/L Na_2SO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L Thiamin HCl, 3 ml/L trace element solution, pH 7.4; trace element solution: 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $MnSO_4{\cdot}H_2O$, 13.9 g/L $FeCl_3{\cdot}6H_2O$, 10.05 g/L EDTA Titriplex III, 0.18 g/L $CoCl_2{\cdot}6\ H_2O$, 0.662 g/L $CaCl_2{\cdot}2H_2O$. As carbon source either OC/AC, OC_{FANC}/AC_{FANC} or OC_{SPE}/AC_{SPE} was used. Acetate was used as reference carbon source. For OC or AC Wilm's KP_i agar plates, 1 g OC or 1 ml AC, respectively, was added to 100 ml Wilm's KP_i medium containing 1.5% agar. To enable the comparison of different cultivations with OC_{FANC}/OC_{SPE}, acetate concentrations were adjusted using 200 g/L sodium acetate solution, which was required due to initial dilution by cold-water extraction.

2.4 | Sampling, sample processing, and analytics

All photometric measurements were taken using a spectrophotometer (WPA CO8000 Cell density meter, Biochrom Ltd., Cambridge, UK) by measuring the optical density at $\lambda = 600$ nm (OD_{600}).

The remaining sample was centrifuged (12,500×*g*, 15 min) to obtain cell-free supernatant for acetate detection. The concentration of acetate was determined from the supernatant samples using acetate assay kit (Enztech yellow line, R-Biopharm AG, Darmstadt, Germany) according to the manufacturers' instructions.

2.5 | Data analysis

All experimental data were obtained at least as duplicates from at least two individual biological experimental setups

and shown as average values ± standard deviation. Data were fitted using a logistic equation with four parameters in a scientific data analysis and graphing software (Sigma Plot 13.0, Systat, San Jose, USA). Obtained data and the resulting fits were used to calculate the specific growth rate μ (hr). Substrate-to-biomass yields $Y_{X/S}$ (g/g) were calculated using the contained amount of acetate.

3 | RESULTS AND DISCUSSION

3.1 | Pretreatment of bio-oil fractions

The major component of the two bio-oil fractions produced in the bioliq® process is acetic acid (Table 1), which can be a potential carbon source for some microorganisms (Berg et al., 2002; Gerstmeir et al., 2003; Li et al., 2016; Lian et al., 2012). However, solids and pyrolytic lignin in OC, and residual oil in AC, as well as reactive and inhibitory substances make their application as microbial substrates very challenging. By the first pretreatment step, water-soluble substances such as organic acids and sugars within the viscous OC were extracted by cold-water extraction to be separated from residual bio-oil, pyrolytic lignin, and solids. The resulting fractions were sterile-filtered and referred to as OC_{FANC}, respectively, AC_{FANC} (Figure 1). The acetate concentration of OC_{FANC} and AC_{FANC} was about 6 g/L, respectively, 43 g/L. The relatively low acetate content in OC_{FANC} originates from its cold-water extraction resulting in a dilution of water-soluble substances.

A more purified fraction was achieved by solid phase extraction. Here, neutral and aromatic compounds within OC_{FANC} and AC_{FANC} were reduced. These fractions are called OC_{SPE}, respectively, AC_{SPE} (Figure 1) and exhibit acetate concentrations of about 5 g/L and 42 g/L. There is a small decrease in acetate content when treated with solid phase extraction.

To see the effect of pretreatments on the reactivity of bio-oil fractions, incubations of cell-free medium supplemented with bio-oil fractions were performed and the drift of optical density was measured during incubation and depicted in Figure 2. Measuring the OD_{600}, no utilizable results could be obtained due to strong drift and variability between individual measurements as well as control measurements. Incubation of cell-free medium with OC_{FANC} or AC_{FANC} (Figure 2a) revealed significant increase in OD_{600} reaching values up to 2 when incubated on 5 g/L acetate in

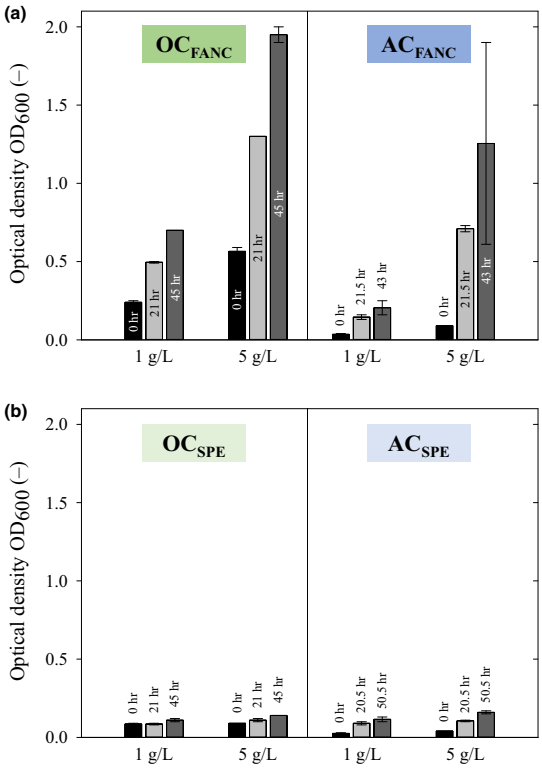

FIGURE 2 Time-course of optical density OD_{600} in cell-free medium supplemented with bio-oil fractions. (a) organic condensate and aqueous condensate after filtration, autoclaving, neutralization, and centrifugation (OC_{FANC} (left), AC_{FANC} (right)), (b) organic condensate and aqueous condensate after solid phase extraction (OC_{SPE} (left), AC_{SPE} (right))

FIGURE 3 Photograph of organic condensate (OC). High viscosity of OC and the formation of elastic-type particles interferes the measurement of OD_{600}, dry weight, and cell counting with microscopy

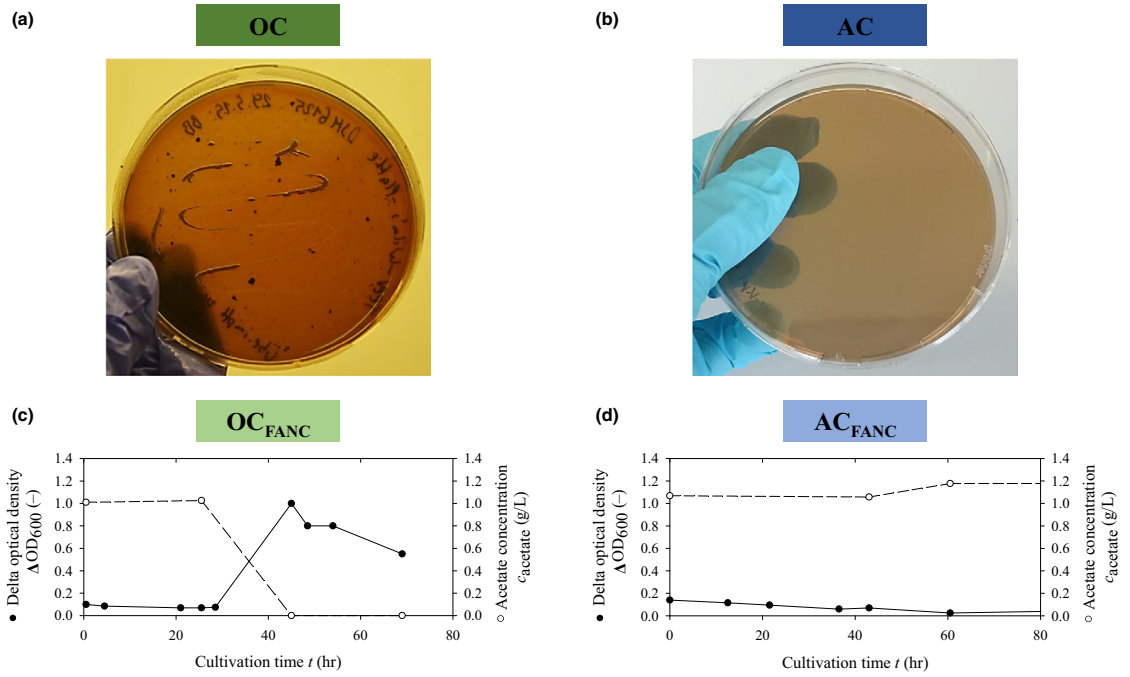

FIGURE 4 Colony formation and growth of *P. putida* KT2440 on organic condensate (OC) and aqueous condensate (AC). Growth on agar plates: Untreated OC (a) and AC (b) in Wilm's KP_i agar plates. Growth in liquid medium in shake flasks: OC and AC subjected to filtration, autoclaving, neutralization, and centrifugation (FANC) and adjusted to 1 g/L acetate: OC_{FANC} (c) and AC_{FANC} (d) cultivations

OC_{FANC} for 45 hr. Cell-free medium with OC_{SPE} or AC_{SPE} (Figure 2b) only changed slightly. Noticeable is the comparably high-standard deviation of high concentrations of AC_{FANC} and long incubation period (Figure 2a).

3.2 | Cultivations with OC or AC as sole carbon source

Experiments with untreated bio-oil fractions in culture suspensions severely hinder analytical procedure and the determination of the optical density of the culture. Especially, the high viscosity of OC and the formation of elastic-type particles interferes with the measurement of the OD_{600} (Figure 3) as well as the dry weight. Also, cell counting with microscopy is difficult, because it is not feasible to differentiate between small OC particles and *P. putida* cells (Figure 3). But the major problem is the reactivity of bio-oil fractions. During cultivation, the culture suspension containing bio-oil fractions is getting darker and particles precipitate. This reactivity leads to strong variations within an OD_{600} measurement as well as dry weight determination making it impossible to verify biomass formation. Growth of *P. putida* on untreated OC was only detected using Wilm's KP_i agar plates containing OC as carbon source (Figure 4a). The bacteria seem to form biofilm to overcome the inhibitors within OC making growth possible (Khiyami, Pometto, & Brown, 2005). Growth of *P. putida* is neither detectable on liquid nor on solid medium containing AC as sole carbon source (Figure 4b). This may be explained by the increased bioavailability of growth inhibitory water-soluble compounds in AC such as furans and methanol (Table 2).

3.3 | Cultivation with OC_{FANC} or AC_{FANC} as sole carbon source

By mixing OC with water and removing the pyrolytic lignin, first enables the detection of growth in liquid medium (Figure 4c). This could be explained by the fact that the mixing with water caused a dilution, which leads to a lower concentration of inhibitors within OC_{FANC}, but also to a lower concentration of microbial accessible components such as acetate (only 5 g/L). This low concentration of acetate was adjusted to a final acetate concentration of 20 g/L acetate in OC_{FANC}, which again resulted in a slight dilution of the inhibitors.

The time-course of the OD_{600} of cultivations with 1 g/L acetate content in OC_{FANC} or AC_{FANC} as sole carbon source is depicted in Figure 4c,d. Shown values for OD_{600} are mean OD_{600} values of the medium inoculated with

TABLE 2 Summary of process parameters of different cultivations using untreated and pretreated bio-oil fractions

	$c_{acetate}$ (g/L)	growth	μ_{max} (hr^{-1})	$Y_{X/S}$ (g/g)	$^{max}OD_{600}$ (–)
Acetate	1	✓	0.6–0.9	0.33	0.9
	3	✓			2.7
	5	✓			4.2
OC		only on agar plate	n.a.	n.a.	n.a.
AC		×	n.a.	n.a.	n.a.
OC_{FANC}	1	✓	0.1–0.2	0.32	1.0
	3	×	n.a.	n.a.	n.a.
	5	×	n.a.	n.a.	n.a.
AC_{FANC}	1	×	n.a.	n.a.	n.a.
	3	×	n.a.	n.a.	n.a.
	5	×	n.a.	n.a.	n.a.
OC_{SPE}	1	✓	0.1–0.3	0.26	0.9
	3	✓			1.8
	5	×	n.a.	n.a.	n.a.
AC_{SPE}	1	✓	0.1–0.2	0.46	1.3
	3	×	n.a.	n.a.	n.a.
	5	×	n.a.	n.a.	n.a.

Note. Cultivation on acetate is given as reference. n.a.: not available.

microorganisms subtracted by values obtained from medium without inoculation (Supporting information Figure S1). *P. putida* was able to grow on a final concentration of 1 g/L acetate content in OC_{FANC} up to a ΔOD_{600} of 1 in 45 hr (Figure 4c). At the maximal $\Delta OD_{600,}$ acetate was completely depleted. For higher concentrations of about 5 g/L acetate in OC_{FANC} growth was not detectable. OD_{600} values of the medium with 5 g/L acetate content in OC_{FANC} inoculated with microorganisms and medium without inoculation increased equally (Supporting information Figure S1) and the acetate concentration remained unchanged during cultivation (data not shown).

As AC was not mixed with water to obtain AC_{FANC}, there is no dilution and the concentration of inhibitors within AC_{FANC} is much higher compared to AC_{FANC}. In addition, no adjustment to a higher acetate concentration has to be performed, because the acetate concentration in AC_{FANC} is ~42 g/L. Therefore, it is obvious that growth on AC_{FANC} poses a significantly higher challenge than on OC_{FANC}. Cultivations with 1 g/L acetate content in AC_{FANC} as sole carbon source showed no increase of ΔOD_{600} values and no decrease in acetate concentration over time (Figure 4d). Also, OD_{600} values of medium containing 5 g/L acetate in AC_{FANC} inoculated with microorganisms and medium without inoculation increased equally (Supporting information Figure S1), while the acetate concentration remained unchanged during cultivation (data not shown).

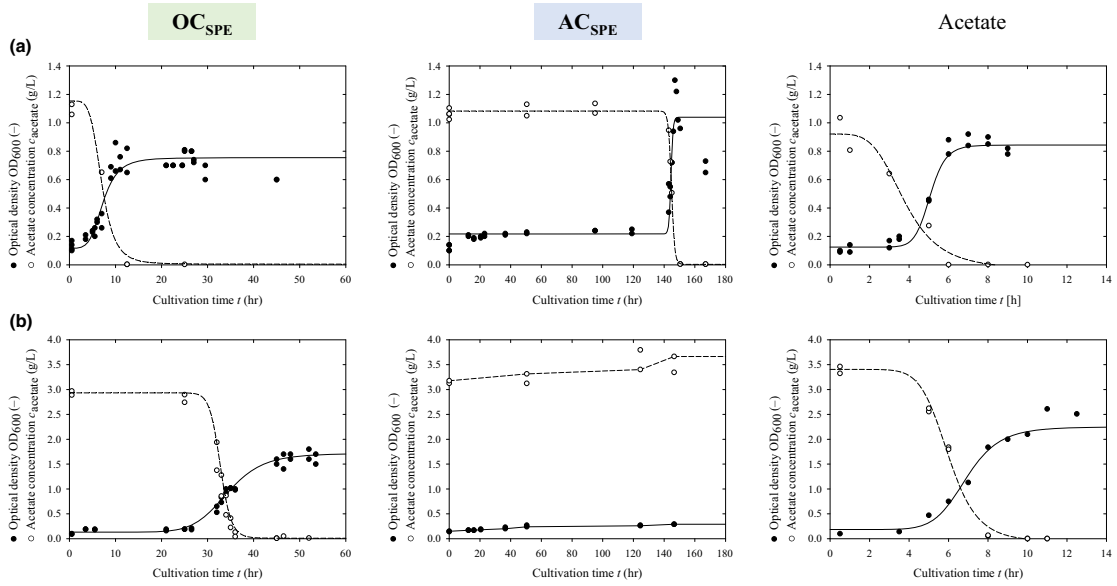

FIGURE 5 Cultivations of *P. putida* KT2440 on solid phase extracted bio-oil fractions. Cultivations were performed with OC_{SPE} or AC_{SPE} containing medium equivalent to concentrations of 1 g/L acetate (a) and 3 g/L acetate (b) as well as reference cultures using pure acetate, respectively

As shown in Figure 2a, there is still a strong reactivity of the pretreated bio-oil fractions OC_{FANC} and AC_{FANC} during incubations, which results in a significant drift in optical density and hinders common analytical methods.

3.4 | Cultivation with OC_{SPE} or AC_{SPE} as sole carbon source

SPE facilitates more stable bio-oil fractions (Figure 2b). Using bio-oil fractions after SPE as sole carbon source, *P. putida* was able to grow on higher concentrations of OC (up to 3 g/L acetate content in OC_{SPE}), as well as the first time on AC (1 g/L acetate content in AC_{SPE}) (Figure 5). This is attributed to the employed SPE, which retains aromatic and hydrophobic compounds that have been reported to interfere with microbial growth (Chi et al., 2013; Jarboe et al., 2011; Lian et al., 2010; Prosen et al., 1993).

Considering the different concentrations of $OC_{SPE,}$ it is obvious that the higher the concentration of OC_{SPE} the longer the duration of the lag-phase is (Figure 5). Cultivations with higher acetate concentrations (5 g/L) in OC_{SPE} showed no increase in OD_{600} and no decrease in acetate during cultivation after incubation time of up to 7 days (data not shown). Using AC_{SPE} as sole carbon source, *P. putida* grew on 1 g/L acetate content in AC_{SPE} up to a maximal OD_{600} of 1.3 within 147 hr (Figure 5a). While a total of 1 g/L acetate in AC_{SPE} cultivations was depleted completely, acetate concentrations of AC_{SPE} cultivations with higher AC_{SPE} concentrations remain constant during cultivation supposing that *P. putida* is not able to grow on higher AC_{SPE} concentrations (Figure 5b). Reference cultivations on different acetate concentrations without any addition of bio-oil fractions were performed for comparison (Figure 5). Growth of *P. putida* was detected up to an acetate concentration of 5 g/L.

3.5 | Comparison of cultivation parameters

As a first assessment of potential suitability of pretreated bio-oil fractions as carbon sources for biotechnology, process parameters of different cultivations using untreated and pretreated bio-oil fractions are compared (Table 2). While maximum growth rates on pretreated bio-oil fractions are in a similar range (0.1–0.3 hr^{-1}), in contrast, the maximal growth rate of cultivations on pure acetate is significantly higher (0.6–0.9 hr^{-1}). This may mainly be attributed to the fact that many inhibitory substances (Table 1) are not or only partially removed by the applied pretreatment methods. Substrate-to-biomass conversion yields Y_{XlS} are in a similar range for cultivation on pure acetate, OC_{FANC} and OC_{SPE} of ~0.3 g/g. Cultivation on $AC_{SPE,}$ however, shows significantly higher yields of 0.46 g/g. This can mainly be attributed to the fact that AC_{SPE} as the aqueous fraction of pyrolysis contains additional potential carbon sources such as propionate, formate, and acetol compared to OC_{SPE}.

Using bio-oil as an alternative carbon source for microbial cultivation is very challenging due to its instability and highly complex composition along with strong adverse effects on microbial growth and existing analytical procedures. In this study, it was shown that SPE may be a suitable tool to obtain stable bio-oil fractions with less inhibitory substances which facilitates growth of *P. putida* KT2440 on low-sugar bio-oil fractions.

This provides a first step toward establishing bio-oil as a feedstock for microbial valorization. Future work should address direct bioconversion and investigate appropriate fractionating condensation designs and conditions.

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ORCID

Nicolaus Dahmen http://orcid.org/0000-0002-5877-4776
Marius Henkel http://orcid.org/0000-0002-5343-9661

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Evaluation of small organic acids present in fast pyrolysis bio-oil from lignocellulose as feedstocks for bacterial bioconversion

Stefanie Arnold | **Tatjana Tews** | **Manuel Kiefer** | **Marius Henkel** | **Rudolf Hausmann**

Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Stuttgart, Germany

Correspondence
Marius Henkel, Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany.
Email: Marius.Henkel@uni-hohenheim.de

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Abstract

Small organic acids derived from fast pyrolysis of lignocellulosic biomass represent a significant proportion of microbially accessible carbon in bio-oil. However, using bio-oil for microbial cultivation is a highly challenging task due to its strong adverse effects on microbial growth as well as its complex composition. In this study, the main small organic acids present in bio-oil as acetate, formate and propionate were evaluated with respect to their suitability as feedstocks for bacterial growth. For this purpose, the growth behavior of four biotechnological production hosts—*Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Corynebacterium glutamicum*—was quantified and compared. The bacteria were cultivated on single acids and mixtures of acids in different concentrations and evaluated using common biotechnological efficiency parameters. In addition, cultivation experiments on pretreated fast pyrolysis-derived bio-oil fractions were performed with respect to the suitability of the bacterial strains to tolerate inhibitory substances. Results suggest that both *P. putida* and *C. glutamicum* metabolize acetate—the major small organic acid generated during fast pyrolysis of lignocellulosic biomass—as sole carbon source over a wide concentration range, are able to grow on mixtures of small organic acids present in bio-oil and can, to a limited extent, tolerate the highly toxic inhibitory substances within bio-oil. This work provides an important step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks and thus contributes to establishing efficient bioprocesses within a future bioeconomy.

KEYWORDS

Bacillus, bacteria, bioeconomy, biomass, bio-oil, *Corynebacterium*, *E. coli*, fast pyrolysis, lignocellulose, *Pseudomonas*

Abbreviations: AC_{SPE}, aqueous condensate after solid phase extraction; OC_{SPE}, organic condensate after solid phase extraction.

1 | INTRODUCTION

Today, most value-added products of industrial biotechnology are produced by bioconversion of glucose as carbon source (Wendisch et al., 2016). However, within the frame of a biobased economy, the search for alternative sustainable carbon sources is driven by a competing application of sugars in human and animal nutrition.

As an abundant, renewable and no direct food and feed competing resource, lignocellulosic biomass is being considered as a potential carbon source for biotechnological production of sustainable value-added compounds. Lignocellulose is the major structural component of plants and is primarily composed of cellulose, hemicellulose, and lignin, which are strongly connected with each other. Therefore, the conversion of lignocellulosic biomass into value-added compounds requires a prior effective degradation of this complex structure. For this, a different separation and degradation methods such as mechanical, chemical, biological, physicochemical, thermochemical, and/or biochemical methods (e.g., reviewed in Anwar, Gulfraz, & Irshad, 2014; Barakat, Vries, & Rouau, 2013; Kumar & Sharma, 2017) are normally used. Fast pyrolysis, which is a thermochemical degradation method, converts lignocellulosic biomass into liquid bio-oil. This complex mixture is principally composed of water and many organic components including pyrolytic sugars, small organic acids, phenolic compounds, alcohols, furans, aldehydes, and ketones (Arnold, Moss, Henkel, & Hausmann, 2017; Mohan, Pittman, & Steele, 2006; Piskorz, Scott, & Radlein, 1988).

Of special interest for biotechnological processes are fast pyrolysis-derived sugars and small organic acids, as they constitute a significant proportion of microbially accessible carbon in bio-oil. To date, the main focus of biotechnological application of bio-oil has been microbial utilization of pyrolytic sugars as carbon source (Chi et al., 2013; Kim, Um, Bott, & Woo, 2015; Layton, Ajjarapu, Choi, & Jarboe, 2011; Lian et al., 2010; Lian, Garcia-Perez, & Chen, 2013; Linger, Hobdey, Franden, Fulk, & Beckham, 2016; Prosen, Radlein, Piskorz, Scott, & Legge, 1993; Wang et al., 2012). Less is known about bioconversion of small organic acids derived from fast pyrolysis of lignocellulosic biomass (Dang et al., 2014; Lian, Garcia-Perez, Coates, Wu, & Chen, 2012; Liang et al., 2013), especially regarding their bacterial valorization.

Acetic acid, primarily generated during deacetylation of hemicellulose, is with about 10 wt% the most common organic acid in bio-oil (Table 1) and has gained attention as a low-cost alternative carbon source for microbial cultivation with no direct competition with food supplies. The ability of using C2 compounds as carbon sources and converting them to anaplerotic compounds is found in some plants and microorganisms. Kornberg and Krebs (1957) identified two enzymes, isocitrate lyase (ICL; gene: *aceA*) and malate synthase (MS; gene: *aceB*), which, together with reactions of the tricarboxylic acid (TCA) cycle, enable *Escherichia coli* to grow on acetate as sole carbon source. This modified TCA cycle is named glyoxylate cycle. While glucose is taken up in bacteria by the phosphotransferase system resulting in intracellular glucose-6-phosphate, which enters the glycolytic pathway and TCA cycle to generate NADH + H^+, $FADH_2$, GTP and biosynthetic precursors, acetate is directly transported into the cell, where it is activated to acetyl coenzyme A (acetyl-CoA) by either the ACK-PTA (acetate kinase, encoded by *ack* and phosphotransacetylase, encoded by *pta*) or acetyl-CoA synthase (ACS, encoded by *acs*) pathway, both being ATP-dependent reactions (Figure 1). Different studies showed that ICL and MS are active and their genes *aceA* and *aceB* are highly upregulated when acetate is present

TABLE 1 Proportion of small organic acids in bio-oil obtained from different biomass raw materials

	Small organic acids (wt%)		
Biomass raw material	**Acetic acid**	**Formic acid**	**Propionic acid**
Beech wood (Demirbas, 2007)	12.6	0.72	0.49
Spruce wood (Demirbas, 2007)	12.2	0.92	0.57
Olive husk (Demirbas, 2007)	10.2	0.87	0.59
Hazelnut shell (Demirbas, 2007)	11.4	0.99	0.64
Pine sawdust (Bertero, Puente, & Sedran, 2012)	5.58	0.95	0.30
Mesquite sawdust (Bertero et al., 2012)	4.33	1.07	0.19
Wheat shell (Bertero et al., 2012)	6.18	1.86	0.97
Rice husk (Guo, Wang, Wang, Guo, & Luo, 2011)	13.49	n.a.	1.65
Pine (Guo et al., 2010)	17.1	n.a.	0.88
Average	10.34	1.05	0.70

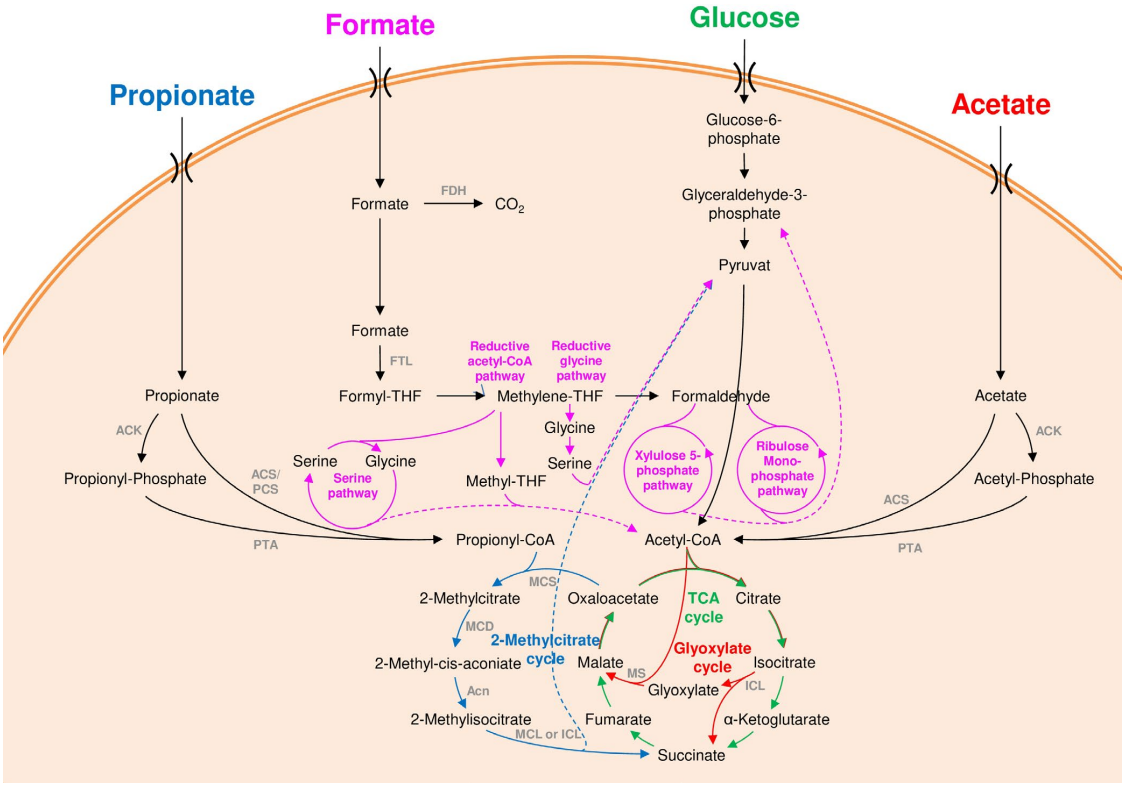

FIGURE 1 Schematic overview of the basic metabolic pathways of glucose, acetate, formate, and propionate

as the sole carbon source for microbial growth (Collins & Kornberg, 1960; Gerstmeir, Cramer, Dangel, Schaffer, & Eikmanns, 2004; Hayashi et al., 2002; Kornberg, 1966; Kornberg & Madsen, 1958; Reinscheid, Eikmanns, & Sahm, 1994a, 1994b; Wendisch, Graaf, Sahm, & Eikmanns, 2000; Wendisch et al., 1997). However, many bacteria lack the key glyoxylate cycle enzyme ICL. It has been reported that these bacteria utilize alternate pathways for acetate assimilation (Alber, Spanheimer, Ebenau-Jehle, & Fuchs, 2006; Ensign, 2006; Ivanovsky, Krasilnikova, & Berg, 1997; Khomyakova, Bukmez, Thomas, Erb, & Berg, 2011). Besides acetic acid, other small organic acids found in bio-oil serve as additional carbon sources for microbial growth.

Formic acid, one of the simplest organic compounds, is typically present in bio-oil with about 1 wt% (Table 1). In the past, bioconversion of formic acid was mainly investigated in methylotrophs and lithoautotrophs (Li et al., 2012; Lidstrom & Stirling, 1990; Schauer & Ferry, 1980; Torella et al., 2015; Tremblay & Zhang, 2015). The metabolization of formate is either by formate oxidation or formate assimilation (Bar-Even, 2016; Bar-Even, Noor, Flamholz, & Milo, 2013; Siegel et al., 2015; Tai & Zhang, 2015). Oxidation of formate relies on an NAD(P)H-dependent formate dehydrogenase (FDH) that catalyzes the oxidation of formate to CO_2. Little is known about growth behavior and yields during bacterial growth. However, in yeasts and fungi, during co-metabolization of formate, increased yields of the primary carbon source were reported as opposed to low biomass yield during growth on formate as a sole source of carbon (Bruinenberg, Jonker, Dijken, & Scheffers, 1985; Harris, Krogt, Gulik, Dijken, & Pronk, 2007; Liu et al., 2017). In contrast, formate assimilation pathways all start with the entry of formate into the cellular metabolism through formate–tetrahydrofolate (THF) ligase that catalyzes the ATP-dependent production of formyl-THF from formate and THF, which is then reduced to methylene–THF. Methylene–THF can be converted into a central metabolism intermediate such as acetyl-CoA, pyruvate, or glyceraldehyde 3-phosphate via five possible pathways: (a) serine pathway, (b) reductive acetyl-CoA pathway, (c) reductive glycine pathway, (d) xylulose 5-phosphate pathway, and (e) ribulose monophosphate pathway (Figure 1; Bar-Even et al., 2013). Considering low biomass yields during metabolization of formate, synthetic pathways have been designed to tackle this challenge (Bar-Even, 2016; Siegel et al., 2015).

Propionic acid, also generated during fast pyrolysis of lignocellulosic biomass, is the third common small organic

acid found in bio-oil with about 0.7 wt% (Table 1). Based on various studies, it is assumed that in most bacteria, yeast, and filamentous fungi, the 2-methylcitrate cycle is the main pathway used to oxidize propionate to pyruvate (Brock, Maerker, Schutz, Volker, & Buckel, 2002; Claes, Pühler, & Kalinowski, 2002; Horswill & Escalante-Semerena, 1999; London, Allen, Gabel, & DeRose, 1999; Miyakoshi, Uchiyama, Someya, Satoh, & Tabuchi, 1987; Pronk, Linden-Beuman, Verduyn, Scheffers, & Dijken, 1994; Tabuchi, Serizawa, & Uchiyama, 1974; Textor et al., 1997). As for acetate, propionate is first activated to propionyl-CoA by either ACS (encoded by *acs*) and propionyl-CoA synthase (encoded by *prpE*) or a sequential reaction of phosphorylation of propionate catalyzed by ACK and PTA (Figure 1). The first step of the 2-methylcitrate cycle involves the condensation of propionyl-CoA with oxaloacetate to 2-methylcitrate by methylcitrate synthase (encoded by *prpC*) followed by an isomerization reaction that converts 2-methylcitrate to 2-methylisocitrate via a 2-methyl-cis-aconiate intermediate. This step is accomplished by a methylcitrate dehydratase (encoded by *prpD*) followed by subsequent hydration catalyzed by an aconitase (encoded by *acn*). 2-Methylisocitrate is finally cleaved by 2-methylisocitrate lyase (encoded by *prpB*) or by ISL into pyruvate and succinate.

For the utilization of fast pyrolysis-derived small organic acids as feedstocks for bacterial growth, it is important that the applied bacterial strain is able to use acetate, formate, and propionate as carbon and/or energy source, can handle different concentrations thereof, as well as tolerates inhibitory substances that are present in bio-oils. This work aims to assess four major bacterial production systems of industrial biotechnology—*E. coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Corynebacterium glutamicum*—for their suitability of utilizing fast pyrolysis-derived small organic acids as carbon sources.

2 | MATERIALS AND METHODS

2.1 | Chemicals

All chemicals used in the current study were either purchased from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) unless stated otherwise.

2.2 | Strains

The four applied industrial bacterial strains *E. coli* K12 DSM498, *P. putida* KT2440 DSM6125, *B. subtilis* DSM10^{T}, and *C. glutamicum* DSM20300 were obtained from the DSMZ strain collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) and were conserved as glycerol stocks at −80°C.

2.3 | Culture media and conditions

All precultures were performed in a 250 ml baffled shake flask containing 25 ml LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) and 100 µl of the respective glycerol stock. The shake flasks were incubated at 30°C, respectively 37°C for *E. coli*, and 120 rpm in a shaking incubator (Newbrunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany) for 18 hr. To obtain cell solutions free of medium components, the precultures were centrifuged for 4 min at 4,550 *g* and 20°C (Heraeus X3R, Thermo Fisher Scientific GmbH, Braunschweig, Germany) and the cell pellets were washed with 1 vol. sterile 9 g/L NaCl solution, centrifuged again and resuspended in fresh sterile 9 g/L NaCl solution. The cell-NaCl solutions were then diluted at 0.1 OD_{600} in 50 ml of the respective main culture medium in 500 ml baffled shake flasks and incubated in a shaking incubator at 120 rpm and 30°C, (37°C for *E. coli*). Each strain was cultivated in its suitable culture medium.

Escherichia coli was cultivated in a minimal medium (Henkel et al., 2015) consisting of the following components: 5 g/L NH_4Cl, 14.6 g/L K_2HPO_4, 3.6 g/L $Na_2HPO_4 \cdot 2H_2O$, 5.6 g/L Na_2SO_4, 0.54 g/L $MgCl_2 \cdot 6H_2O$ complexed with 1 g/L sodium citrate $\cdot 2H_2O$, 0.26 g/L $CaCl_2 \cdot 2H_2O$, 0.01 g/L thiamin, 3 ml/L trace element solution (trace element solution: 25.8 g/L sodium citrate $\cdot 2H_2O$, 0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.1 g/L $MnSO_4 \cdot H_2O$, 8.35 g/L $FeCl_3 \cdot 6H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 0.01 g/L $NiCl_2 \cdot 6H_2O$, 0.016 g/L $NaMoO_4 \cdot 2H_2O$, 0.001 g/L $Na_2SeO_3 \cdot 5H_2O$). The pH value was adjusted to 7.0.

The employed main culture medium for cultivation of *P. putida* was adapted Wilm's KP_i medium (Wilms et al., 2001): 6.58 g/L K_2HPO_4, 1.64 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 2 g/L Na_2SO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.05 g/L thiamin HCl, 3 ml/L trace element solution (trace element solution: 0.18 g/L $ZnSO_4 \cdot 7H_2O$, 0.16 g/L $CuSO_4 \cdot 5H_2O$, 0.1 g/L $MnSO_4 \cdot H_2O$, 13.9 g/L $FeCl_3 \cdot 6H_2O$, 10.05 g/L EDTA Titriplex III, 0.18 g/L $CoCl_2 \cdot 6H_2O$, 0.662 g/L $CaCl_2 \cdot 2H_2O$). The pH value of the buffer solution was adjusted to 7.4.

Bacillus subtilis was cultivated in a modified mineral salt medium based on culture medium of Cooper, Macdonald, Duff, and Kosaric (1981) (Willenbacher et al., 2014): 0.1 M NH_4Cl, 0.03 M KH_2PO_4, 0.04 M $Na_2HPO_4 \cdot 2H_2O$, 8.0×10^{-4} M $MgSO_4 \cdot 7H_2O$, 1 ml/L trace element solution (4.0×10^{-3} M Na_2 EDTA $\cdot 2H_2O$, 7.0×10^{-3} M $CaCl_2$, 4.0×10^{-3} M $FeSO_4 \cdot 7H_2O$, 1.0×10^{-3} M $MnSO_4 \cdot H_2O$). The pH value of the buffer solution was adjusted to 7.0.

Modified CGXII minimal medium (Eikmanns, Metzger, Reinscheid, Kircher, & Sahm, 1991; Keilhauer, Eggeling, & Sahm, 1993; Lange et al., 2017) was used for cultivation of *C. glutamicum* containing the following components: 5 g/L $(NH_4)_2SO_4$, 5 g/L urea, 21 g/L 3-(*N*-morpholino)

propanesulphonic acid, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $CaCl_2 \cdot 2H_2O$, 10 mg/L $MnSO_4 \cdot H_2O$, 16.4 g/L $FeSO_4 \cdot 7H_2O$, 1 mg/L $ZnSO_4 \cdot 7H_2O$, 0.2 mg/L $CuSO_4 \cdot 5H_2O$, 0.02 mg/L $NiCl_2 \cdot 6H_2O$, 0.2 mg/L biotin. The pH value was adjusted to 7.2.

2.4 | Carbon sources

The effect of small organic acids on bacterial growth was investigated by adding them aseptically to the respective culture medium. The following stock solutions of carbon sources were prepared and sterilized by autoclaving (glucose), respectively, by sterile filtration: 500 g/L glucose stock solution, 200 g/L acetate stock solution, 200 g/L propionate stock solution, and 200 g/L formate stock solution. Cultivations on glucose were performed with a final glucose concentration of 10 g/L. Up to seven concentrations of acetate (1, 5, 10, 15, 20, 25, and 30 g/L) were investigated. Propionate and formate were added to the culture medium at final concentrations of 1, 5, and 10 g/L. Cultivations with mixtures of organic acids were performed with 10 g/L acetate supplemented with either 1 g/L formate or 1 g/L propionate or with 1 g/L formate and 1 g/L propionate.

Cultivation experiments with lignocellulosic degradation products as sole carbon sources were accomplished by using pretreated bio-oil fractions: organic condensate after solid phase extraction (OC_{SPE}) and aqueous condensate after solid phase extraction (AC_{SPE}) as described previously (Arnold, Moss, Dahmen, Henkel, & Hausmann, 2018). Briefly, bio-oil fractions were added to the medium and adjusted to final concentrations of 1, 3, and 5 g/L acetate.

2.5 | Analytical methods

During cultivation, samples of 1 ml culture were taken to monitor cell growth and consumption of carbon source. The optical density at $\lambda = 600$ nm (OD_{600}) was determined using a spectrophotometer (UV-3100 PC; VWR GmbH, Darmstadt, Germany). After measuring the OD_{600}, the samples were centrifuged for 15 min at 15,500 *g* and 4°C (Centrifuge 5430 R; Eppendorf AG) to obtain the cell-free supernatant for glucose and acetate determination. Consumption of glucose and acetate as carbon sources was measured from the supernatant samples using enzymatic assay kits (Enztech yellow line; R-Biopharm AG, Darmstadt, Germany) following the manufacturer's instructions. The photometric measurements were performed using a spectrophotometer (WPA CO8000 Cell density meter; Biochrom Ltd., Cambridge, UK).

A correlation between OD_{600} and biomass concentration c_{BM} (g/L) (c_{BM} (g/L) = OD_{600} (-)/α (g/L)) was determined for each bacterial strain by measuring the cell dry mass in shake flask cultivations of the respective culture medium containing 10 g/L glucose. The correlation factor α is 3.3 g/L for *E. coli*, 2.6 g/L for *P. putida*, 4.2 g/L for *B. subtilis*, and 3.7 g/L for *C. glutamicum*.

2.6 | Data analysis

Experimental data were obtained as duplicates from at least two individual biological experiments, resulting in at least four individual measurements for all datasets. Data were fitted applying a logistic four-parameter equation in a scientific data analysis and graphing software (Sigma Plot 13.0; Systat, San Jose, CA, USA). Obtained data as well as the resulting fits were used to calculate the specific growth rate μ (per hour) and maximum substrate-to-biomass yields $Y_{X/S}^{ac,max}$ (g/g).

3 | RESULTS

3.1 | Characterization of growth on acetate

For comparison of growth behavior on acetate to growth on glucose as a sole source of carbon, growth experiments with culture media containing 10 g/L glucose or 10 g/L acetate, respectively, were performed exemplarily and depicted in Figure 2. As expected, addition of 10 g/L acetate promoted growth to a lower cell density, resulted in lower specific growth rates, as well as longer lag-phases than the same concentration of glucose. While *E. coli* achieved a maximal biomass concentration of 4.84 g/L after 18.5 hr on glucose, the maximal biomass yield on 10 g/L acetate was reached after 53 hr and is about 10 times less (0.52 g/L; Figure 2a). The acetate concentration decreased slightly during cultivation and at a concentration of about 8 g/L left the growth stagnated until the cultivation was finally stopped after 80 hr. On glucose as sole carbon source, the biomass production of *P. putida* reached a maximum of 6.92 g/L after 16 hr cultivation time and a maximal growth rate of 0.77 per hour (Figure 2b). The biomass yield of *P. putida* on acetate is approximately half of that achieved on glucose while simultaneously displaying longer lag times. The growth of *C. glutamicum* on 10 g/L glucose or 10 g/L acetate, respectively, is shown in Figure 2d. The addition of 10 g/L acetate to the culture medium of *C. glutamicum* led to a maximal growth rate of 0.55 per hour and generated biomass of 3.24 g/L within 44 hr, whereas cultivations on glucose reached maximal growth rates of 0.65 per hour and biomass yields of 5.1 g/L. *Bacillus subtilis* yielded a biomass of 2.2 g/L and a specific growth rate of 0.7 per hour on glucose (Figure 2c). After the addition of acetate, neither an increase in biomass nor a decrease in acetate was observed.

The effect of acetate on bacterial growth was investigated by using up to seven different acetate concentrations (1, 5, 10, 15, 20, 25, and 30 g/L; Table 2). In the presence of acetate as sole carbon source in the culture medium, all

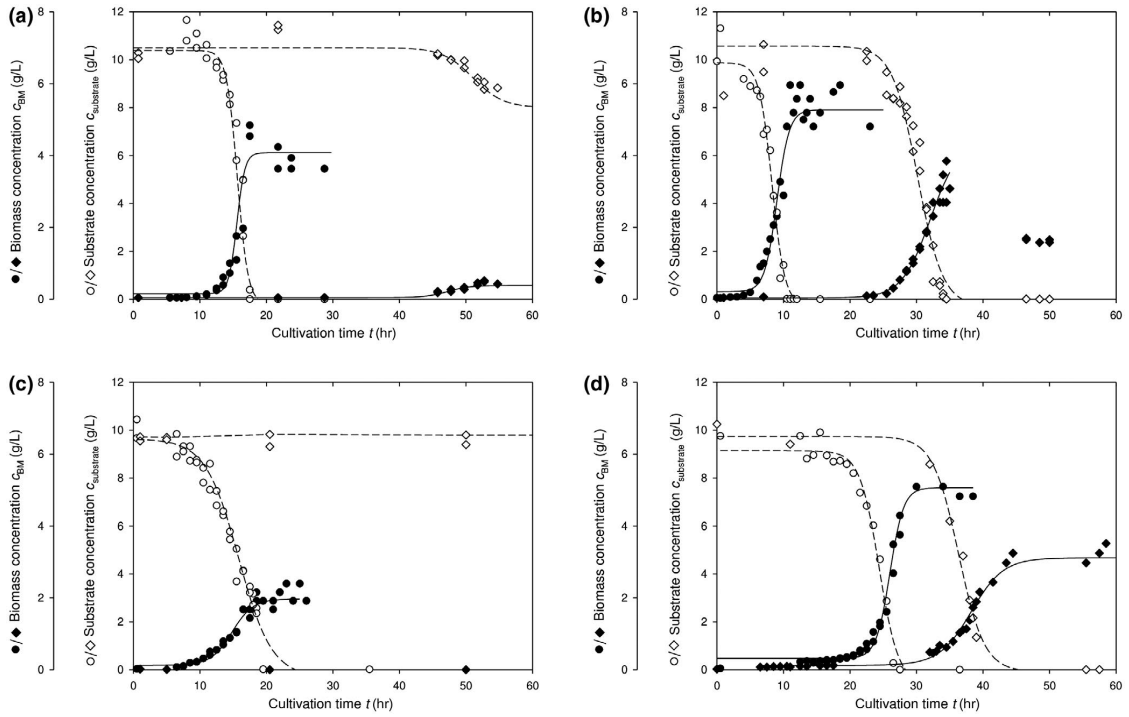

FIGURE 2 Cultivation of (a) *Escherichia coli* K12, (b) *Pseudomonas putida* KT2440, (c) *Bacillus subtilis* DSM 10^T, and (d) *Corynebacterium glutamicum* DSM 20300 on 10 g/L glucose (●—biomass concentration on glucose/○—glucose concentration) or on 10 g/L acetate (◆—biomass concentration on acetate/◇—acetate concentration) as sole carbon source

applied bacterial strains were able to grow, except *B. subtilis*. Cultivation experiments with *E. coli* on different acetate concentrations showed that the concentration of acetate is not completely depleted but rather decreases around 12% during cultivation. The overall maximal biomass concentration of 0.52 g/L was achieved in cultivation with 10 g/L acetate. The growth behavior of *E. coli* on different acetate concentrations indicates that *E. coli* is inhibited by acetate. The biomass production of *P. putida* increased with increasing acetate concentrations from 0.35 g/L for 1 g/L acetate to 6.54 g/L for 15 g/L acetate in the culture medium. For all four concentrations (1, 5, 10, and 15 g/L), acetate was completely depleted during cultivation. For higher acetate concentration, growth was not detectable. In contrast, *C. glutamicum* was able to grow on all seven acetate concentrations by complete depletion of acetate. The biomass concentration increased from 0.27 to 10 g/L. In the presence of 30 g/L acetate, *C. glutamicum* was not able to grow in each cultivation experiment. While *C. glutamicum* showed a long stationary phase for all acetate concentrations in which the number of cells remained the same (Figure 2d right), the cells of *P. putida* started decreasing immediately after reaching the maximal biomass concentration resulting in a peak in the cell growth curve (Figure 2b right).

3.2 | Characterization of growth on further pyrolytic small organic acids

Further growth experiments were performed by using three different concentrations (1, 5, and 10 g/L) of other small organic acids such as formate and propionate, which can be present in bio-oil.

Cultivations on formate revealed that all applied bacterial strains are unable to grow on formate as sole carbon source. There was no increase in the biomass concentration.

Growth experiments with propionate as sole carbon source showed that only *P. putida* is able to grow on all three applied propionate concentrations. The biomass concentration increased from 0.58 to 4.62 g/L with increased propionate concentrations. *Escherichia coli* showed growth on 1 g/L propionate; however, only at higher concentrations of propionate, biomass growth could be measured. During the entire cultivation, a maximal biomass yield (1.12 g/L) was achieved on 5 g/L propionate in the culture medium. With higher propionate concentrations of 10 g/L, *E. coli* reached a maximal biomass concentration of 0.61 g/L, which afterward decreased until the cultivation was finally stopped after 200 hr. *Corynebacterium glutamicum* was only able to grow on 1 g/L propionate reaching a maximal biomass

TABLE 2 Summary of parameters of cultivations with small organic acids (✓ growth; ✗ no growth; n.a., not applicable; n.d., not determined)

Strain:		*E. coli* K12 DSM498				*P. putida* KT2440 DSM6125				*B. subtilis* DSM10^T				*C. glutamicum* DSM20300			
Small organic acid	c (g/L)	Growth	c_{BM}^{max} (g/L)	μ_{max} (per hour)	$Y_{XS}^{ac,max}$ (g/g)	Growth	c_{BM}^{max} (g/L)	μ_{max} (per hour)	$Y_{XS}^{ac,max}$ (g/g)	Growth	c_{BM}^{max} (g/L)	μ_{max} (per hour)	$Y_{XS}^{ac,max}$ (g/g)	Growth	c_{BM}^{max} (g/L)	μ_{max} (per hour)	$Y_{XS}^{ac,max}$ (g/g)
Acetate (A)	1	✓	n.a.	n.a.	n.a.	✓	0.35	0.60	0.31	✗	0	0	0	✓	0.27	0.32	0.26
	5	✓	0.33	0.40	0.17	✓	1.62	0.77	0.28	✗	0	0	0	✓	1.89	0.61	0.37
	10	✓	0.52	0.33	0.37	✓	3.85	0.68	0.31	✗	0	0	0	✓	3.24	0.55	0.32
	15	✓	0.48	0.15	0.39	✓	6.54	0.46	0.44	✗	0	0	0	✓	6.22	0.48	0.39
	20	✓	0.30	0.08	0.16	✗	0	0	0	✗	0	0	0	✓	7.84	0.39	0.37
	25	n.d.	n.a.	n.a.	n.a.	n.d.	n.a.	n.a.	n.a.	n.d.	n.a.	n.a.	n.a.	✓	9.91	0.22	0.36
	30	n.d.	n.a.	n.a.	n.a.	n.d.	n.a.	n.a.	n.a.	n.d.	n.a.	n.a.	n.a.	✓	10.0	0.16	0.33
Formate (F)	1	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.
	5	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.
	10	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.	✗	0	0	n.d.
Propionate (P)	1	✓	n.a.	n.a.	n.d.	✓	0.58	0.64	n.d.	✗	0	0	n.d.	✓	0.24	0.15	n.d.
	5	✓	1.12	0.12	n.d.	✓	2.12	0.54	n.d.	✗	0	0	n.d.	✗	0	0	n.d.
	10	✓	0.58	0.07	n.d.	✓	4.62	0.39	n.d.	✗	0	0	n.d.	n.d.	n.a.	n.a.	n.d.
AF	10 + 1	✓	0.28	0.17	0.13	✓	2.69	0.36	0.24	✗	0	0	0	✓	2.70	0.23	0.27
AP	10 + 1	✓	0.42	0.34	0.27	✓	3.85	0.55	0.32	✗	0	0	0	✓	3.51	0.34	0.35
AFP	10 + 1 + 1	✓	0.32	0.21	0.36	✓	3.85	0.59	0.30	✗	0	0	0	✓	3.51	0.39	0.26

concentration of 0.24 g/L, whereas *B. subtilis* did not grow on any of the applied propionate concentrations.

3.3 | Characterization of growth on mixtures of small organic acids

The effect of mixtures of small organic acids on the growth of the applied bacterial strains was investigated by adding different combinations of small organic acids to the culture medium. According to typical proportions of small organic acids in bio-oil (Table 1), acetate was used as the major carbon source with a concentration of 10 g/L in the culture medium, whereas formate and propionate were applied as supplementary carbon sources with a respective concentration of 1 g/L. Figure 3 shows the cell growth of the four bacterial strains on three different mixtures of small organic acids: (a) AF = 10 g/L acetate + 1 g/L formate, (b) AP = 10 g/L acetate + 1 g/L propionate, and (c) AFP = 10 g/L acetate + 1 g/L formate + 1 g/L propionate. As for cultivations on 10 g/L acetate as sole carbon source, the growth behavior on mixtures of small organic acids is mostly similar. While *E. coli*, *P. putida*, and *C. glutamicum* are able to grow on all three mixtures, *B. subtilis* shows no growth on any small organic acid mixtures (Table 2). Similar to cultivations with acetate, *P. putida* and *C. glutamicum* completely depleted acetate during the cultivations, whereas the acetate concentration only decreased by around 22% during cultivation of *E. coli* on small organic mixtures.

The results also confirmed that formate has an inhibitory effect on bacterial growth. While maximal biomass concentrations of 0.52 g/L for *E. coli*, 3.85 g/L for *P. putida*, and 3.24 g/L for *C. glutamicum* on 10 g/L acetate were achieved, maximal biomass production on AF was about 30% less. Interestingly, when cultivations were performed on AFP mixtures, formate has only an adverse effect on *E. coli*.

Cultivations of *P. putida* on AP and AFP reached the same values for the process parameters as on 10 g/L acetate. *Corynebacterium glutamicum* achieved slightly higher

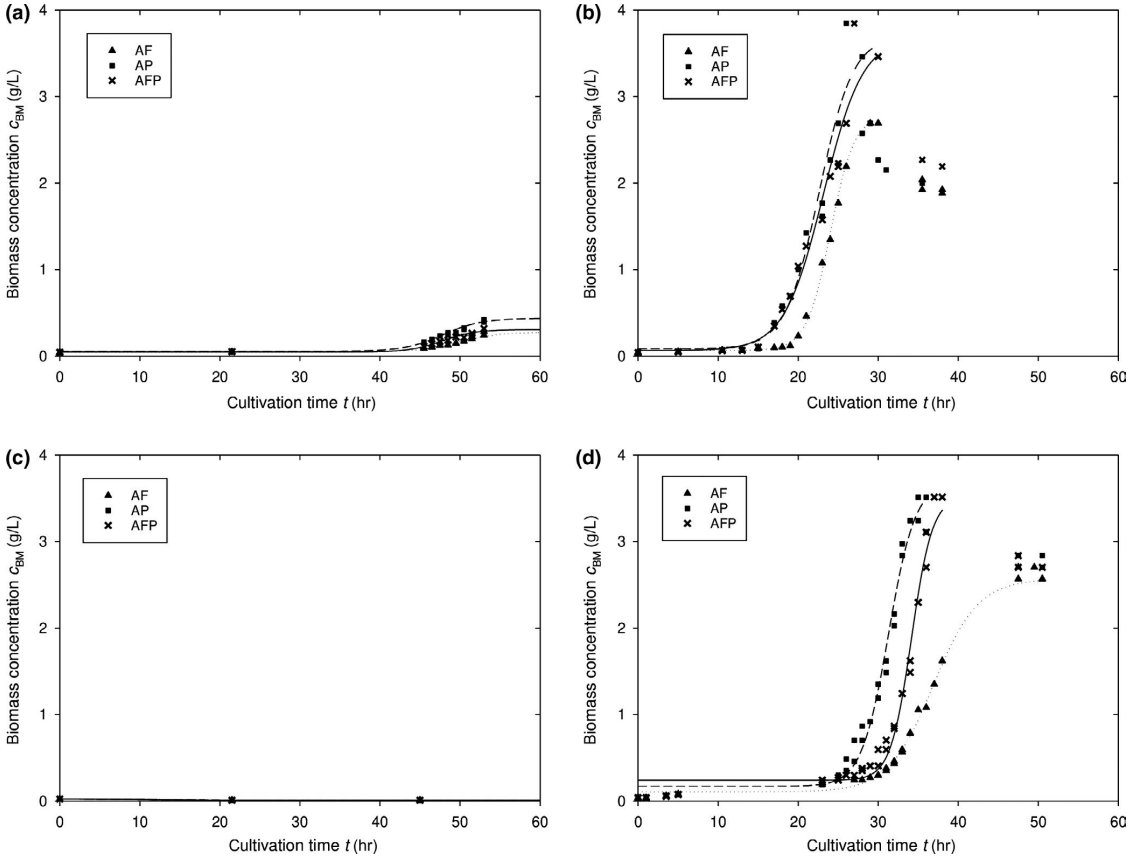

FIGURE 3 Cultivation of (a) *Escherichia coli* K12, (b) *Pseudomonas putida* KT2440, (c) *Bacillus subtilis* DSM 10^T, and (d) *Corynebacterium glutamicum* DSM 20300 on different mixtures of small organic acids (▲ AF = 10 g/L acetate + 1 g/L formate; ■ AP = 10 g/L acetate + 1 g/L propionate; **x** AFP = 10 g/L acetate + 1 g/L formate + 1 g/L propionate)

growth parameters on AP and AFP than on 10 g/L acetate. The lag-phase of both bacterial strains is briefer on small organic mixtures compared to 10 g/L acetate (Figures 2 and 3).

3.4 | Characterization of growth on pretreated bio-oil fractions

In order to evaluate the suitability of the bacterial strains for their growth on bio-oil and tolerance toward inhibitory substances within this complex mixture, cultivation experiments on pretreated bio-oil fractions were performed. Figure 4 shows the maximal biomass concentrations of the four bacterial strains achieved by cultivation on the two pretreated bio-oil fractions OC_{SPE} and AC_{SPE} applied in three different concentrations. The concentrations of the fractions were adjusted based on acetate as described previously (Arnold et al., 2018). According to the other growth experiments, no growth was detectable for *B. subtilis* (Figure 4c). Although *E. coli* was able to grow on OC_{SPE} with 1 g/L acetate with complete depletion of acetate, the maximal biomass concentration of 0.27 g/L was only reached after 216 hr (Figure 4a). Both *P. putida* and *C. glutamicum* showed growth on all adjusted concentrations of OC_{SPE} with a simultaneous decrease in acetate until it was completely depleted (Figure 4b,d). The comparison of the two strains revealed that *C. glutamicum* achieves higher biomass yields than *P. putida*. However, *P. putida* requires less cultivation time for reaching maximal biomass concentration compared to *C. glutamicum*, e.g. cultivations on OC_{SPE} with 5 g/L acetate resulted in a maximal biomass production of 1.62 g/L for *C. glutamicum* after 169 hr and 0.85 g/L for *P. putida* within only 70 hr. Additionally, *P. putida* is the only bacterial strain investigated that is able to grow on the pretreated aqueous bio-oil fraction AC_{SPE} (Figure 4b). Cultivations of *P. putida* on AC_{SPE} with 1 g/L acetate resulted in a maximal biomass production of 0.5 g/L, which is even higher than the biomass yield on OC_{SPE} with 1 g/L acetate amounting to a maximum of 0.33 g/L. However, the cultivation time for achieving maximal biomass concentration on AC_{SPE} is 15 times longer than on OC_{SPE} containing 1 g/L acetate.

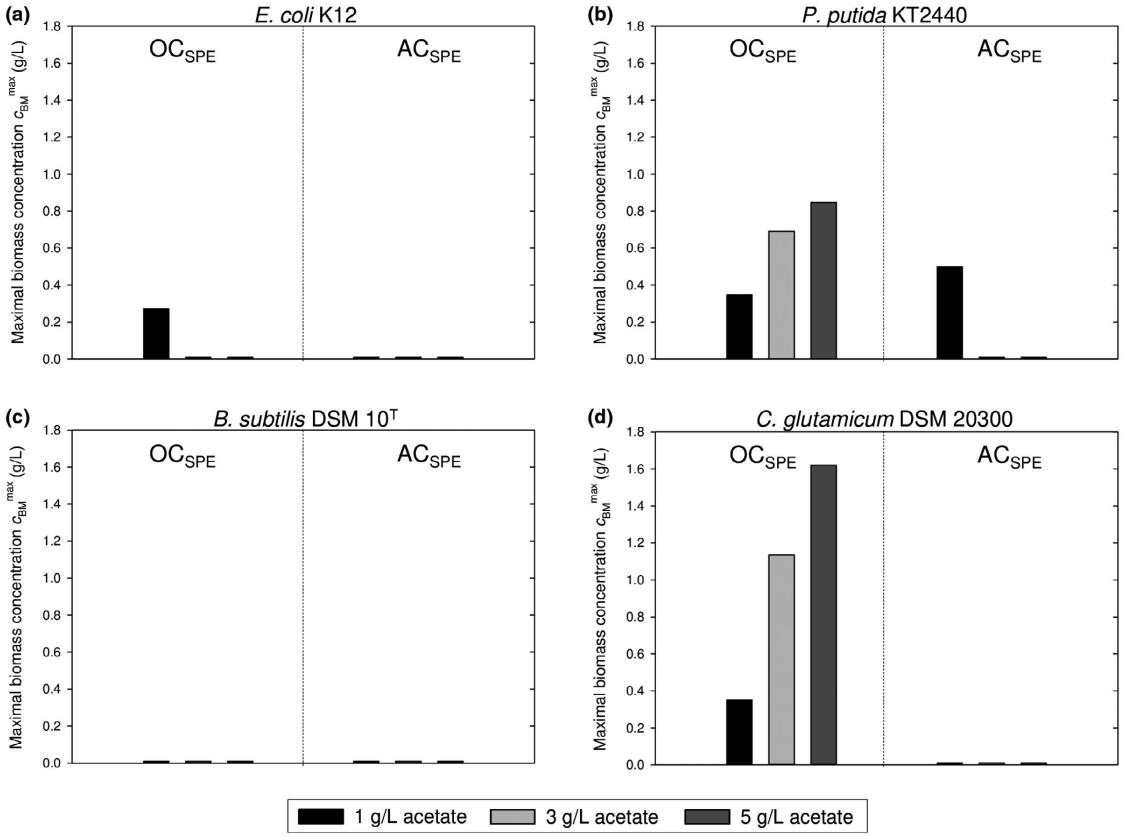

FIGURE 4 Cultivation of (a) *Escherichia coli* K12, (b) *Pseudomonas putida* KT2440, (c) *Bacillus subtilis* DSM 10^T, and (d) *Corynebacterium glutamicum* DSM 20300 on pretreated bio-oil fractions OC_{SPE} and AC_{SPE} containing 1, 3, or 5 g/L acetate

4 | DISCUSSION

The ability to use small organic acids derived from fast pyrolysis of lignocellulosic biomass is an interesting and nonconventional possibility to establish efficient bioprocesses within a future bioeconomy. However, knowledge of the effect and valorization of small organic acids on bacterial growth is scarce. Therefore, this study evaluated four bacterial strains for their suitability using different concentrations of acetate, mixtures of small organic acids, as well as pretreated bio-oil fractions as carbon source for their growth. Results show that *E. coli*, *P. putida*, and *C. glutamicum* are able to grow on acetate as a sole source of carbon, whereas growth of *B. subtilis* on acetate was not detectable. This behavior can potentially be attributed to the glyoxylate cycle (Berg, Tymoczko, & Stryer, 2002). While *E. coli*, *P. putida*, and *C. glutamicum* possess the key enzymes of the glyoxylate cycle and thus are able to grow on acetate as sole carbon source (Cortay et al., 1989; Gerstmeir et al., 2003; Kornberg & Krebs, 1957; Reinscheid, Eikmanns, & Sahm, 1994a; Sudarsan, Dethlefsen, Blank, Siemann-Herzberg, & Schmid, 2014; Wendisch et al., 2000), *B. subtilis* lacks enzymes required for the glyoxylate cycle (Freese & Fortnagel, 1969). To enable growth of *B. subtilis* on acetate, Kabisch et al. (2013) transferred an operon encoding the two key enzymes of the glyoxylate cycle, ISL and MS, from *B. licheniformis*.

Although *E. coli* has the ability to grow on acetate as sole carbon source, it is obvious that its growth is affected by acetate in the growth medium. Previous reports suggest that this effect is potentially caused by an interfering effect of acetate on methionine biosynthesis leading to the accumulation of homocysteine, which inhibits the growth of *E. coli* (Roe, O'Byrne, McLaggan, & Booth, 2002). Roe et al. (2002) demonstrated that even 0.5 g/L acetate in the culture medium reduced the specific growth rate of *E. coli* by 50% compared to an uninhibited control. This inhibition can be relieved by adding methionine to the culture medium (Han, Hong, & Lim, 1993).

With regard to the effect of acetate on *P. putida*, results suggest that *P. putida* can efficiently grow on acetate up to a concentration of 15 g/L. Higher acetate concentrations, however, have an adverse effect on cell growth. The phenomenon of subsequent decrease in biomass after reaching maximal biomass concentration was also observed by Arias-Barrau, Olivera, Sandoval, Naharro, and Luengo (2006).

In agreement with previous studies (Claes et al., 2002; Gerstmeir et al., 2003; Jolkver et al., 2009; Wendisch et al., 2000), the findings of this study show that *C. glutamicum* can grow on acetate. The specific growth rates achieved in our cultivations were, within typical margin of error, in the same range compared to those of Wendisch et al. (2000). In contrast to the previous studies, higher acetate concentrations were tested and it was shown that *C. glutamicum* can grow on up to 30 g/L acetate. The results reveal that the growth rate decreases with increasing acetate concentrations, which was in the past attributed to its effects on the transmembrane pH gradient (Baronofsky, Schreurs, & Kashket, 1984). Growth inhibition of *C. glutamicum* by acetate was initially observed at a concentration of 30 g/L acetate. Although *C. glutamicum* was able to grow on 30 g/L acetate reaching maximal biomass concentrations of 10 g/L, growth was not detectable for every cultivation experiment with 30 g/L acetate.

Furthermore, the effect of formate and propionate on cell growth was investigated. It was found that none of the tested bacterial strains was able to grow on formate as sole carbon source, whereas propionate was used for cell growth by some bacteria. The fact that the strains cannot use formate as a carbon source might be attributed to the lack of metabolic enzymes for C1 metabolism in the genome of these strains (Roca, Rodriguez-Herva, Duque, & Ramos, 2008).

Propionate utilization was observed for *E. coli*, *P. putida*, and *C. glutamicum*. The results are in agreement with other studies, which showed that the methylcitrate cycle is present in these bacterial strains (Claes et al., 2002; Textor et al., 1997).

Cultivations on mixtures of small organic acids revealed that formate has an adverse effect on cell growth. Formate is known as both an energy source and a compound potentially toxic to growth. Under certain conditions, formate can be used as an auxiliary energy substrate and thus has a beneficial effect on cell growth and product formation (Bruinenberg et al., 1985; Harris et al., 2007; Lian et al., 2012). However, depending on cell density, pH, activity of FDH, formate concentration, and physiological state of the cells, formate was also found to have inhibitory effects.

On the basis of growth analysis in the presence of pretreated bio-oil fractions, *P. putida* and *C. glutamicum* were found to be suitable for bioconversion of lignocellulosic-based feedstocks.

Both *P. putida* and *C. glutamicum* were able to grow on all three OC_{SPE} concentrations. However, *C. glutamicum* reached higher maximal biomass concentrations than *P. putida*. This could be attributed to the ability of *C. glutamicum* to grow on a wide variety of carbohydrates and organic acids as single or combined sources of carbon and energy (Arndt, Auchter, Ishige, Wendisch, & Eikmanns, 2008; Claes et al., 2002; Cocaign, Monnet, & Lindley, 1993; Wendisch et al., 2000). In comparison to cultivations on acetate, the results suggest that low concentrations of OC_{SPE} have no significant effect on cell growth, but the higher the OC_{SPE} concentration, the higher the adverse effect on cell growth, especially for *P. putida*. The results suggest that *P. putida* is not able to use all carbon sources present in OC_{SPE} just as efficiently as *C. glutamicum* does. Nevertheless, cultivations on AC_{SPE} revealed that *P. putida* is the only strain able to grow on this bio-oil fraction, even with a higher biomass yield than achieved on OC_{SPE} and 1 g/L acetate.

Pseudomonas putida is known as an organic solvent–tolerant strain, able to degrade and adapt to high concentrations of organic solvents (Inoue & Horikoshi, 1989; Kim & Park, 2014; Nikel & de Lorenzo, 2014; Rojas et al., 2004; Rühl, Schmid, & Blank, 2009; Weber, Ooijkaas, Schemen, Hartmans, & Bont, 1993). It is assumed that AC_{SPE} contains compounds, which are less inhibitory to *P. putida* as well as additional carbon sources for *P. putida*, whereas these substances have an adverse effect on the growth of *C. glutamicum*.

Bio-oil derived from fast pyrolysis of lignocellulosic biomass provides a sustainable resource for biotechnological production of value-added compounds with no direct competition to food and feed. The small organic acid fraction of bio-oil represents a significant proportion of microbially accessible carbon, which is being considered as one potential carbon source for an envisioned future industrial biotechnology. However, due to inhibitory substances as well complex composition, accessing these organic acids as substrates for growth remains a challenging task. The results of this study suggest that both *P. putida* and *C. glutamicum* are able to metabolize acetate as sole carbon source over a wide concentration range and are able to grow on mixtures of the main small organic acids present in bio-oil. Both *P. putida* and *C. glutamicum* show a distinct potential to tolerate inhibitory substances within bio-oil, which provides an important step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks.

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ORCID

Marius Henkel https://orcid.org/0000-0002-5343-9661
Rudolf Hausmann https://orcid.org/0000-0002-2327-7120

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Heterologous rhamnolipid biosynthesis by *P. putida* KT2440 on bio-oil derived small organic acids and fractions

Stefanie Arnold[1†], Marius Henkel[1*†], Janina Wanger[1], Andreas Wittgens[2,3,4], Frank Rosenau[2,3,4] and Rudolf Hausmann[1]

Abstract

In many cases in industrial biotechnology, substrate costs make up a major part of the overall production costs. One strategy to achieve more cost-efficient processes in general is to exploit cheaper sources of substrate. Small organic acids derived from fast pyrolysis of lignocellulosic biomass represent a significant proportion of microbially accessible carbon in bio-oil. However, using bio-oil for microbial cultivation is a highly challenging task due to its strong adverse effects on microbial growth as well as its complex composition. In this study, the suitability of bio-oil as a substrate for industrial biotechnology was investigated with special focus on organic acids. For this purpose, using the example of the genetically engineered, non-pathogenic bacterium *Pseudomonas putida* KT2440 producing mono-rhamnolipids, cultivation on small organic acids derived from fast pyrolysis of lignocellulosic biomass, as well as on bio-oil fractions, was investigated and evaluated. As biosurfactants, rhamnolipids represent a potential bulk product of industrial biotechnology where substitution of traditional carbon sources is of conceivable interest. Results suggest that maximum achievable productivities as well as substrate-to-biomass yields are in a comparable range for glucose, acetate, as well as the mixture of acetate, formate and propionate. Similar yields were obtained for a pretreated bio-oil fraction, which was used as reference real raw material, although with significantly lower titers. As such, the reported process constitutes a proof-of-principle for using bio-oil as a potential cost-effective alternative carbon source in a future bio-based economy.

Keywords: Rhamnolipid, Bio-oil, Pyrolysis, Lignocellulosic biomass, Bioeconomy, Biosurfactant

Introduction

The establishment of alternative feedstocks as sources of carbon for industrial biotechnology is a key goal to achieve cost-efficient and economical bio-processes. A general competition between food and biotechnology has placed lignocellulosic biomass and other related carbon sources into the focus of attention as renewable and sustainable raw materials. As such, these substrates hold a significant economic and ecologic potential for industrial biotechnology. Lignocellulosic biomass mainly consists of cellulose, hemicellulose, and lignin. For its utilization as carbon sources in biotechnological processes a prior degradation step is necessary. A promising degradation method is fast pyrolysis, which converts lignocellulosic biomass in the absence of oxygen into bio-oil (Fig. 1). Bio-oil mainly consists of water, pyrolytic sugars, small organic acids, phenolic compounds, alcohols, furans, aldehydes and ketones (Piskorz et al. 1988; Mohan et al. 2006; Arnold et al. 2017). Beside pyrolytic sugars, small organic acids such as acetic acid, formic acid and propionic acid are of special interest for biotechnological processes (Prosen et al. 1993; Lian et al. 2010, 2012, 2013; Layton et al. 2011; Linger et al. 2016).

Due to the fact that bio-oil is a complex mixture composed of hundreds of compounds, microorganisms have to be found which are able to metabolize pyrolytic carbon

*Correspondence: Marius.Henkel@uni-hohenheim.de
†Stefanie Arnold and Marius Henkel contributed equally to this work
[1] Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany
Full list of author information is available at the end of the article

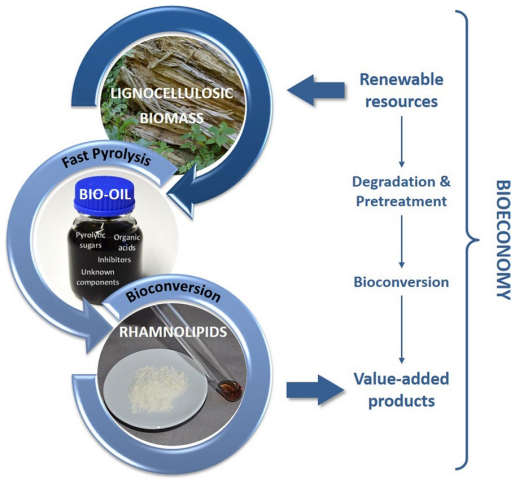

Fig. 1 Bio-oil in the value chain of bioeconomy from renewable resources towards value-added products. Envisioned path of conversion from lignocellulosic biomass to rhamnolipid biosurfactants by pyrolysis intermediate steps for decomposition

sources such as pyrolytic sugars and small organic acids, as well as tolerate inhibitory compounds present in bio-oil. As previous studies emphasized, the Gram-negative, non-pathogenic and organic solvent tolerant soil bacterium *Pseudomonas putida* KT2440 is a promising candidate for bioconversion of bio-oil (Khiyami et al. 2005; Linger et al. 2016; Arnold et al. 2018). Furthermore, this strain is used as a host for heterologous gene expression for different biotechnological purposes (Dammeyer et al. 2011; Wittgens et al. 2011; Nikel and de Lorenzo 2014; Beuker et al. 2016a).

One interesting example of a potential bulk products of white biotechnology which can be produced on lignocellulose are rhamnolipid biosurfactants. Rhamnolipids are surface-active glycolipids which consist of one or two L-rhamnose units which are linked to one or two hydroxy fatty acids. Rhamnolipids are mainly known to be produced by the opportunistic pathogen *Pseudomonas aeruginosa*. Therefore, research in the last decade has addressed rhamnolipid production in nonpathogenic heterologous production hosts (Ochsner et al. 1995; Cha et al. 2008; Wittgens et al. 2011, 2017, 2018; Tiso et al. 2016).

This study describes the heterologous production of mono-rhamnolipids on small organic acids derived from fast pyrolysis of lignocellulosic biomass, as well as on bio-oil fractions by using a genetically engineered *P. putida* KT2440 strain. Both, growth behavior of the engineered *P. putid*a KT 2440 strain and its simultaneous production of rhamnolipids were investigated during cultivation experiments on acetate, formate and propionate as sole carbon sources, on mixtures thereof, as well as on two different bio-oil fractions, to evaluate the potential of bio-oil as an alternative carbon source for heterologous production of rhamnolipid biosurfactants. As such, the investigated system provides a proof-of-principle for using bio-oil as a potential cost-effective alternative carbon source using rhamnolipid biosurfactants as an example for a value-added product (Fig. 1).

Materials and methods

Chemicals and standards

All chemicals used in the current study were either acquired from Carl Roth GmbH (Karlsruhe, Germany) or Sigma-Aldrich (Munich, Germany) if not mentioned otherwise. Mono-rhamnolipid (Rha-C_{10}-C_{10}) standard was obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany) and rhamnolipid standard as mixture of mono- and di-rhamnolipid from Jeneil Biotech Inc. (Saukville, WI, USA).

Strain and plasmid

A genetically engineered *P. putida* KT2440 strain carrying plasmid pSynPro8oT producing mono-rhamnolipids was used for all cultivation experiments. The plasmid harbors genes *rhlAB* required for rhamnolipid biosynthesis as well as a tetracycline selection marker (Beuker et al. 2016a).

Media and cultivation conditions

Conditions of fast-pyrolysis, source and applied setup as well as obtained side-streams are described in Arnold et al. (2018). Cultivations for rhamnolipid production were performed as described by Beuker et al. (2016b).

Pseudomonas putida KT2440 pSynPro8oT_rhlAB was first incubated in 25 mL LB medium (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl; pH 7.0) containing tetracycline (end concentration 20 mg/L) at 30 °C and 120 rpm.

Growth experiments were carried out in 500 mL baffled shake flasks filled 50 mL of adapted Wilm's KP_i medium (Wilms et al. 2001) (6.58 g/L K_2HPO_4, 1.64 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 2 g/L Na_2SO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L Thiamin HCl, 3 mL/L trace element solution (trace element solution: 0.18 g/L $ZnSO_4{\cdot}7H_2O$, 0.16 g/L $CuSO_4{\cdot}5H_2O$, 0.1 g/L $MnSO_4{\cdot}H_2O$, 13.9 g/L $FeCl_3{\cdot}6H_2O$, 10.05 g/L EDTA Titriplex III, 0.18 g/L $CoCl_2{\cdot}6H_2O$, 0.662 g/L $CaCl_2{\cdot}2H_2O$). Tetracycline was added to the media to an end concentration of 20 mg/L. Different concentrations of glucose, acetate, formate, propionate, mixtures of small organic acids, or pretreated bio-oil fractions [organic condensate after solid phase extraction (OC_{SPE}) and aqueous

condensate after solid phase extraction (AC_{SPE})] were added to the medium as carbon source as described previously (Arnold et al. 2018). The main culture medium was inoculated with a starting optical density at 600 nm (OD_{600}) of 0.1 using cells washed in 0.9% NaCl solution.

Analytical methods

Cell growth was monitored by measuring the optical density at $\lambda = 600$ nm (OD_{600}) using a spectrophotometer (UV-3100 PC, VWR GmbH, Darmstadt, Germany). Consumption of glucose and acetate was determined from the supernatant samples using D-glucose, respectively acetate assay kits (Enztech yellow line, R-Biopharm AG, Darmstadt, Germany) following the manufacturers' instructions. Rhamnolipid determination was performed as described previously (Horlamus et al. 2019). All graphical and regression analysis for production rates and yield coefficients was performed using scientific graphing and data analysis software (SigmaPlot 13.0, Systat Software Inc., San Jose, CA). If applicable, four parameter logistic fits for biomass and rhamnolipid concentration were used for calculation (Henkel et al. 2014).

Results

Cultivations of the recombinant strain *P. putida* KT2440 pSynPro8oT_rhlAB were performed on glucose, acetate, and mixtures of small organic acids mainly present in bio-oil (Fig. 2). During the time course of cultivation biomass formation, substrate consumption, rhamnolipid production, as well as specific growth rate (Fig. 2—bottom graph) was investigated for (a) 10 g/L glucose, (b) 5 g/L acetate, and (c) 5 g/L acetate + 1 g/L formate + 1 g/L propionate (AFP). Similar growth rates of approximately 0.4 h^{-1} were achieved and rhamnolipid production was detected in all three cultivation experiments, although with different titers. For further insight into production capacity, yields and potential inhibitory effects additional cultivations were performed at different concentrations of organic acids and mixtures thereof (Table 1).

To compare acetate with glucose different acetate concentrations from 1 to 10 g/L were applied. The maximum concentration range was chosen depending on typical concentrations in bio-oil resulting from different applied raw material and pyrolysis process conditions. An acetate concentration of 10 g/L approximately corresponds to a 1:10 dilution of average bio-oil composition, which, due to its very high viscosity and inhibitory components, is a reasonable working dilution. Results from cultivations up to 5 g/L acetate revealed that similar growth rates ($\mu = 0.38–0.52$ h^{-1}) and yields ($Y_{X|S} = 0.21–0.24$ and $Y_{P|X} = 0.57–0.76$) were reached when compared to cultivations on glucose from this study ($\mu = 0.38$, $Y_{X|S} = 0.26$, $Y_{P|X} = 0.61$) as well as data from literature (Table 1). While growth rate is significantly reduced to 0.15 h^{-1} at acetate concentrations of 10 g/L, results suggest that an inhibitory effect on growth might not necessarily correlate with an inhibition of rhamnolipid biosynthesis which, at a similar yield coefficient $Y_{P|X}$ of 0.72 compared to 0.62, reaches a similar titer above 250 mg/L, both at 5 g/L and 10 g/L acetate. When using acetate as a sole source of carbon, a decrease in biomass can be observed at a certain biomass concentration reducing biomass from 0.8 g/L below 0.5 g/L (Fig. 2b).

Cultivations with formate as sole source of carbon revealed that with the applied system no metabolization of formate was observed. To further assess its inhibitory effect, formate was further used simultaneously with acetate and acetate plus propionate (AP), respectively. A concentration of 1 g/L formate was chosen, which corresponds to a 1:10 dilution of average bio-oil composition.

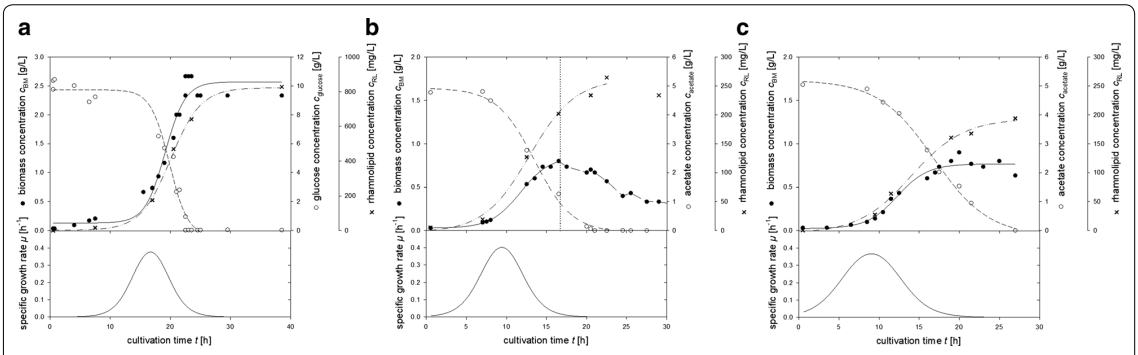

Fig. 2 Time-course of biomass, substrate (glucose or acetate) and mono-rhamnolipid concentration during cultivation of *P. putida* KT2440 carrying plasmid pSynPro8oT_rhlAB on **a** 10 g/L glucose, **b** 5 g/L acetate and on **c** a mixture of small organic acids [5 g/L acetate + 1 g/L formate + 1 g/L propionate (= AFP)] as sole carbon source. Data is presented along with respective specific growth rates

Table 1 Summary of cultivation parameters

Carbon source (CS)	c_{CS} (g/L)	Growth	c_{BM}^{max} (g/L)	μ^{max} (h^{-1})	$Y_{X|S}^{max}$ (g/g)	$Y_{P|X}$ (g/g)	c_{RL}^{max} (mg/L)	q_{RL}^{max} (mg/g/h)	q_{RL}^{avg} (mg/g/h)	References
Sunflower oil										
P. aeruginosa wt, different strains	Excess	✓	9.0–19.0	0.09–0.18	n.d.	0.9–3.1	$8.0–38.0 \times 10^3$	60–190	10–40	Müller et al. (2011)
Glucose										
P. aeruginosa wt, different strains	20–40	✓	2.4–7.7	n.d.	n.d.	0.3–2.3	$0.2–6.1 \times 10^3$	n.d.	12–54	Henkel et al. (2016)
P. putida KT2440 pSynpro8oT_rhlAB	Excess	✓	23.6	0.44	0.24–0.33	n.d.	14.9×10^3	n.d.	18–24	Beuker et al. (2016a, b)
P. putida KT2440 pSynpro8oT_rhlAB	10	✓	2.67	0.38	0.26	0.61	827.7	71.9	42.4	This study
Small organic acids										
Acetate (A)	1	✓	0.20	0.38	0.24	0.76	88.5	74.8	52.7	This study
	3	✓	0.63	0.52	0.23	0.57	191.2	66.7	58.6	
	5	✓	0.80	0.40	0.21	0.62	264.5	62.4	39.2	
	10	✓	0.70	0.15	0.12	0.72	264.2	43.7	23.5	
Formate (F)	1	✖	0	0	n.d.	0	0	0	0	
	3	✖	0	0	n.d.	0	0	0	0	
	5	✖	0	0	n.d.	0	0	0	0	
Propionate (P)	1	✓	0.23	0.25	n.d.	0.90	119.4	74.7	62.4	
	3	✓	0.73	0.24	n.d.	0.47	184.0	49.8	53.9	
	5	✓	0.67	0.16	n.d.	0.61	212.3	19.9	24.7	
	10	✓	2.57	0.21	n.d.	0.07	93.7	n.a.	n.a.	
Mixtures of small organic acids										
AF	5+1	✓	0.63	0.49	0.18	0.56	185.9	56.9	40.4	
AP	5+1	✓	0.87	0.50	0.21	0.53	239.7	80.6	40.8	
AFP	5+1+1	✓	0.90	0.37	0.25	0.41	193.9	72.1	26.6	
Bio-oil fractions										
OC_{SPE}	1[a]	✓	0.27	0.39	0.24	0.48	72.5	28.4	n.a.	
	3[a]	✓	0.37	0.20	0.10	0.43	89.4	2.8	1.8	
	5[a]	✖	0	0	n.a.	0	0	0	0	
AC_{SPE}	1[a]	✖	0	0	n.a.	0	0	0	0	
	3[a]	✖	0	0	n.a.	0	0	0	0	
	5[a]	✖	0	0	n.a.	0	0	0	0	

[a] The bio-oil fractions were added in three different concentrations adjusted according to acetate concentration

✓ growth, ✖ no growth observed, *n.a.* not applicable, *n.d.* not determined

At the applied concentration, formate does not seem to have a negative effect on either growth or product formation (Table 1).

Growth rates were reduced to approximately 50% ($\mu = 0.16–0.25\ h^{-1}$) compared to acetate when using propionate in different concentrations as sole carbon source. An adverse effect on rhamnolipid biosynthesis was observed at propionate concentration between 3 and 5 g/L (Table 1). Despite comparably high biomass production on 10 g/L propionate with a maximal biomass of 2.57 g/L, the maximal rhamnolipid concentration was reduced from 212.3 mg/L for 5 g/L propionate to 93.7 mg/L for 10 g/L propionate. Propionate was further studied as an additional carbon source with acetate and acetate plus formate (AF), respectively. The concentration of 1 g/L propionate was applied, which also corresponds approximately the concentration of a 1:10 diluted average bio-oil composition. Results suggest that, in the applied concentrations, propionate, comparable to formate, has no negative effect on growth and product formation (Table 1).

Growth and rhamnolipid production was also possible on mixtures of small organic acids with a corresponding concentration of a 1:10 diluted average bio-oil composition. Formate and propionate had no observable negative effect on either growth or rhamnolipid formation.

Furthermore, growth and rhamnolipid production was investigated using pretreated bio-oil fractions as sole carbon sources. The bio-oil fractions OC_{SPE} and AC_{SPE} were added in three different concentrations of acetate up to 5 g/L adjusted as described by Arnold et al. (2018). While growth was detectable on OC_{SPE} with concentrations of up to 3 g/L (Fig. 3), a significant inhibitory effect on growth is already observed at this concentration, resulting in a reduction of specific growth rate from 0.39 h^{-1} to almost half the value of 0.20 h^{-1}. In contrast, no biomass formation was observed when AC_{SPE} was used as sole carbon source. To reach a final concentration of acetate of 3 g/L in OC_{SPE}, an approximate 1:35 dilution of average bio-oil would be required. This is a significantly higher dilution compared to application of artificial substrate mixtures, however, is explained by a high number and amount of inhibitory and unknown components in bio-oil. Compared to cultivations on acetate this further shows an inhibitory effect on rhamnolipid biosynthesis, where addition of 3 g/L acetate in OC_{SPE} does not significantly increase rhamnolipid titers of 72.5 mg/L observed at 1 g/L acetate in OC_{SPE}.

Discussion

In summary, the results suggest that acetate represents a potentially suitable carbon source for rhamnolipid production. In direct comparison to rhamnolipid production obtained with the same strain and genetic construct on glucose as a sole source of carbon from this study as well as previous studies (Beuker et al. 2016a, b), both maximum specific rhamnolipid production rate as well as average production rate are generally in

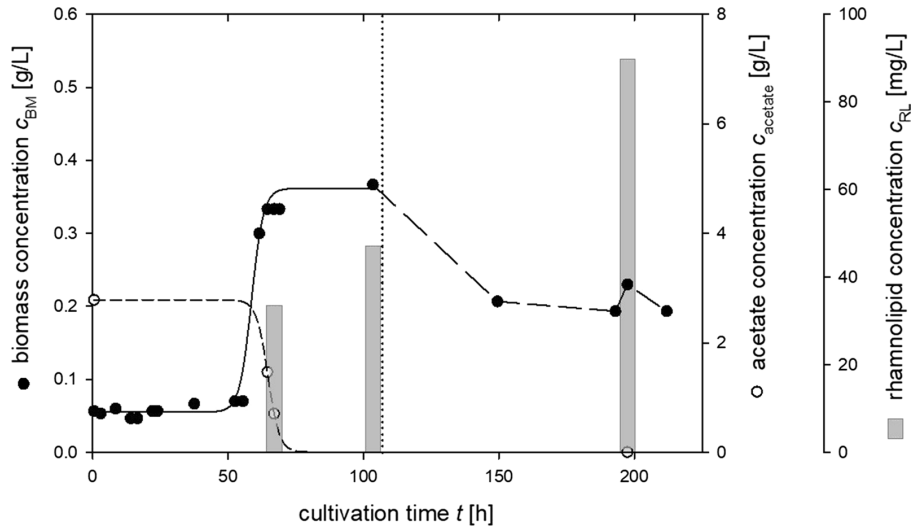

Fig. 3 Time-course of biomass (filled circles), acetate (empty circles) and mono-rhamnolipid concentration (bars) during cultivation of *P. putida* KT2440 carrying plasmid pSynPro8oT_rhlAB on organic condensate of bio-oil pyrolysis process after solid phase extraction (OC_{SPE}) containing 3 g/L acetate

a similar range between approx. 45–75 mg/(g h) and 25–45 mg/(g h) (Table 1). It should be noted however that while maximum specific rhamnolipid production rates on glucose and low concentrations of acetate are highly similar [71.9 mg/(g h) on glucose versus 74.8 mg/(g h) on 1 g/L acetate], this is not the case for average production rates, which are approx. half in the study used for comparison with the same biological system (18–24 mg/(g h) versus 42 mg/(g h). This is due to the fact that Beuker et al. (2016a, b) developed a fed-batch process over 3 days which was aimed for high-titer production, and thus not optimized for average or maximum production rates. For reference, high-yield processes with *P. aeruginosa* using plant or vegetable oil are further used for comparison. Obtained titers of up to 38 g/L as well as maximum production rates of up to 190 mg/(g h) are significantly higher than for both glucose as well as organic acids. Average production rates, on the other hand, are on average lower for *P. aeruginosa* using plant or vegetable oil. This is due to the fact that quorum sensing has a major effect on rhamnolipid biosynthesis in wild-type *P. aeruginosa* thus significantly restricting the window of time during cultivation where rhamnolipid production is observed. However, comparing rhamnolipid production of *P. aeruginosa* on glucose, similar ranges are observed for average production rates up to 54 mg/(g h) compared to *P. putida* KT2440.

While the results confirm that rhamnolipid biosynthesis is possible using bio-oil derived carbon sources, for competitiveness on an economic level, several factors would have to be addressed. These factors include modification of the biocatalyst towards higher rhamnolipid productivity, increased metabolic spectrum to metabolize additional components in bio-oil or by engineering for higher tolerance towards inhibitory components. Furthermore, considering the high amount of different substances with potentially inhibitory threshold concentrations, a fed-batch process with continuous addition of substrate may not be feasible due to accumulation of potentially inhibiting or unmetabolized components. Therefore, appropriate techniques for establishing a bioprocess may include investigation of bioreactor systems with biomass retention and flow-through system of medium such as membrane bioreactor constructions, biofilm reactors or perfusion bioreactor systems. In addition, a modification of the applied plasmid-based expression system containing rhamnolipid biosynthesis genes may lead to increased productivity. These modifications may include studies on promoter activity or stable genomic integration. Furthermore, additional renewable sources of acetate or acetate-containing side-streams such as wastewater from cellulose manufacturing (pyroligneous acid) are interesting targets for future investigation.

In this study, it was shown that acetate represents a potentially suitable substrate for biotechnological production of rhamnolipids. Results show that maximum achievable productivities, as well as substrate-to-biomass yields are in a similar range compared to glucose. Furthermore, bio-oil was used in this study as an example for a renewable source of acetate to investigate its application as an alternative substrate for rhamnolipid production. Owing to its very high viscosity as well as complex composition of inhibitory components, bio-oil has to be pretreated and diluted before application for rhamnolipid production. Cultivations with pretreated bio-oil fractions resulted in lower titers with an onset of inhibitory affects at much lower concentrations than for acetate. However, even though bio-oil represents a challenging substrate for rhamnolipid production, it should be noted that maximum production rates of approximately half of different reference processes could be observed. As such, the reported process constitutes a proof-of-principle for using bio-oil as a potential cost-effective alternative carbon source in a future bio-based economy.

Abbreviations

OC_{SPE}: organic condensate after solid phase extraction; AC_{SPE}: aqueous condensate after solid phase extraction; RL: rhamnolipid; AF: acetate + formate; AP: acetate + propionate; AFP: acetate + formate + propionate.

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Authors' contributions

SA planned and executed the experiments, collected data, created the graphs and drafted the manuscript. MH designed and planned the experiments, created the graphs and drafted the manuscript. JW performed part of the experiments and collected and evaluated corresponding data. AW and FR generated the plasmid pSynPro8_rhlAB and contributed to interpretation of the experiment. RH substantially contributed to conception and design of the conducted experiments. All authors read and approved the final manuscript.

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Availability of data and materials

All obtained data have been included into the manuscript. Please turn to the corresponding author for all other requests.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details
[1] Department of Bioprocess Engineering (150k), Institute of Food Science and Biotechnology, University of Hohenheim, Fruwirthstr. 12, 70599 Stuttgart, Germany. [2] Institute for Pharmaceutical Biotechnology, Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany. [3] Ulm Center for Peptide Pharmaceuticals (U-PEP), Ulm University, Albert-Einstein-Allee 11, 89081 Ulm, Germany. [4] Department Synthesis of Macromolecules, Max-Planck-Institute for Polymer Research Mainz, Ackermannweg 10, 55128 Mainz, Germany.

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Publisher's Note

Chapter **3**

General Discussion

In order to the development of a future bio-based economy, the intention of this thesis was to evaluate the potential and challenges of fast pyrolysis bio-oil derived from lignocellulosic biomass as an alternative and sustainable carbon source for bacterial bioconversion.

Bio-oil is a complex mixture and comprises many different components, among them pyrolytic sugars and small organic acids are particularly interesting as potential carbon sources for microbial processes (Prosen *et al.* 1993, Lian *et al.* 2010, 2012, 2013, Layton *et al.* 2011, Islam *et al.* 2015, Linger *et al.* 2016, Arnold *et al.* 2017). However, direct use of bio-oil as carbon source for microbial cultivation is very challenging due to solids and pyrolytic lignin, as well as reactive and inhibitory substances present in bio-oil. Therefore, different pretreatment strategies of the two bio-oil fractions OC and AC produced in the bioliq® process of ash-rich lignocellulosic biomass were investigated in **Part I** to overcome their unsuitability for common analytical methods and strong inhibitory effects. By the first pretreatment step, water-soluble substances within the viscous OC were extracted by cold-water extraction. To remove solids and residual oil in OC extract, as well as in AC, both fractions were filtrated, autoclaved, neutralized, and centrifuged. In a second pretreatment step, hydrophobic and aromatics compounds that have been reported to interfere with microbial growth (Chi *et al.* 2013, Jarboe *et al.* 2011, Lian *et al.* 2010, Prosen *et al.* 1993) were removed by solid phase extraction resulting in a more purified fraction. The effect of pretreatment on reactivity of bio-oil was investigated by incubation of cell-free medium supplemented with bio-oil fractions and measurement the drift of optical density during incubation. While cell-free medium with OC_{FANC} and AC_{FANC} revealed significant increase in OD_{600}, incubation of OC_{SPE} and AC_{SPE} in cell-free medium only changed slightly suggesting less reactive and more stable bio-oil fractions (**Part I**, 3.1). In order to evaluate the applied pretreatment strategies for microbial valorization of the two bio-oil fractions, the organic solvent tolerant bacterial strain *Pseudomonas*

putida KT2440 was used as a reference system and cultivated on the different untreated and pretreated bio-oil fractions. Growth on untreated bio-oil fractions was only detectable using solid medium with OC as sole carbon source (**Part I**, 3.2). Utilization of bio-oil fractions after the first pretreatment enabled growth in liquid medium containing low concentration of OC_{FANC}, however with significant remaining optical instability (**Part I**, 3.3). Using bio-oil fractions after SPE as sole carbon source, *P. putida* KT2440 was able to grow on higher concentrations of OC, as well as the first time on AC (**Part I**, 3.4). In general, it can be concluded that solid phase extraction is a suitable tool to obtain stable bio-oil fractions with less inhibitory substances enabling appliance of common analytical methods and growth of *P. putida* KT2440 on bio-oil fractions produced by fast pyrolysis of ash-rich lignocellulosic biomass.

Besides convenient pretreatment strategies, it is important that the applied microorganism is able to metabolize pyrolytic sugars and organic acids, as well as tolerates inhibitory substances that are present in bio-oil. Since to date, the main focus of biotechnological application of bio-oil has mainly been on microbial utilization of pyrolytic sugars as carbon source (Chi *et al.* 2013, Kim *et al.* 2015, Layton *et al.* 2011, Lian *et al.* 2010, 2013, Linger *et al.* 2016, Prosen *et al.* 1993, Wang *et al.* 2012), less is known about the effect and valorization of small organic acids present in bio-oil on bacterial growth (Dang *et al.* 2014, Lian *et al.* 2012, Liang *et al.* 2013). Hence, **Part II** addressed the evaluation of main small organic acids present in bio-oil as acetate, formate and propionate with respect to their suitability as feedstocks for bacterial growth. Four biotechnological production hosts *Escherchia coli*, *Pseudomonas putida*, *Bacillus subtilis* and *Corynebacterium glutamicum* were selected and cultivated on different concentrations of single and mixtures of small organic acids. Results confirm that *P. putida*, as well as *C. glutamicum* metabolize acetate – the major small organic acid generated during fast pyrolysis of lignocellulosic biomass – as sole carbon source over a wide concentration range (**Part II**, 3.1) and grow on mixtures of small organic acids present in bio-oil (**Part II**, 3.3). Furthermore, cultivation experiments on pretreated bio-oil fractions OC_{SPE} and AC_{SPE} as described in **Part I** were performed regarding the suitability of the selected bacterial strains to tolerate inhibitory substances within lignocellulosic-based feedstocks (**Part II**, 3.4). *P. putida*, as well as *C. glutamicum* were able to grow on OC_{SPE}, whereas *C. glutamicum* reached higher maximal biomass concentrations than *P. putida*. However, the higher the OC_{SPE} concentration, the higher the adverse effect on cell growth. Cultivations on AC_{SPE} revealed that *P. putida* is the only strain able to grow on this bio-oil frac-

tion. In summary, both *P. putida* and *C. glutamicum* showed a distinct potential to use bio-oil derived small organic acids and tolerate inhibitory substances within bio-oil, which provides an important step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks.

In order to establish a bio-based value-added process chain it is crucial to produce value-added products. Since in **Part II** two promising bacterial strains were detected able to grow on bio-oil fractions, **Part III** dealed with the investigation of growth behavior of a genetically engineered, non-pathogenic bacterium *Pseudomonas putida* KT2440 and its simultaneous heterologous production of rhamnolipid biosurfactants as exemplary value-added product on bio-oil derived small organic acids and pretreated fractions as described in **Part I**. Results show that acetate represents a potentially suitable carbon source for biotechnological production of rhamnolipids. Both maximum achievable productivities and substrate-to-biomass yields are in a similar range compared to glucose. Cultivations with pretreated bio-oil fractions resulted in lower titers with an onset of inhibitory effects at much lower concentrations than for acetate. However, even though bio-oil represents a challenging substrate for rhamnolipid production, it should be noted that maximum production rates of approximately half of different reference processes could be observed. Finally, **Part III** implemented a novel value-added process chain using bio-oil derived small organic acids and fractions to produce rhamnolipid biosurfactants as exemplary value-added product.

In conclusion, this thesis shows that microbial valorization of bio-oil remains a challenging task due to its highly complex and variable composition, as well as its adverse effects on microbial growth and issues with analytical procedures. To enhance future prospects of using bio-oil as feedstock for microbial bioconversion, additional knowledge regarding microbial metabolic requirements, bioprocess fundamentals, and pyrolysis process conditions is needed. For competitiveness on an economic level, future work should address several factors including modification of biocatalysts towards increased metabolic spectrum to metabolize additional components in bio-oil or by metabolic engineering for higher tolerance towards typically inhibitors such as furans, 5-hydroxymethyl, furfural or aromatic compounds. Furthermore, considering the high amount of different substances with potentially inhibitory threshold concentrations, a fed-batch process with continuous addition of substrate may not be feasible due to accumulation of potentially inhibiting or unmetabolized components. Therefore, appropriate techniques for establishing a bioprocess may include investigation of bioreactor systems with

biomass retention and flow-through system of medium such as membrane bioreactor constructions, biofilm reactors or perfusion bioreactor systems. A further option is the production of tailor made bio-oils by adaptation of pyrolysis process conditions and fractional condensation.

Despite this challenging feedstock, this thesis depicts a proof of concept by outlining a potential biorefinery route for microbial valorization of bio-oil and its unexploited side streams by using appropriate pretreatment strategies to obtain stable bio-oil with less inhibitory substances. It provides a step in search of suitable bacterial strains for bioconversion of lignocellulosic-based feedstocks into value-added products and thus contributes to establishing bioprocesses within a future bioeconomy.

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Abbreviations

A	acetate
AC	aqueous condensate
aceA	gene encoding isocitrate lyase
aceB	gene encoding malate synthase
acetyl-CoA	acetyl-coenzyme A
AC_{FANC}	aqueous condensate after filtration, autoclaving, neutralization and centrifugation
ACK	acetate kinase
ack	gene encoding acetate kinase
Acn	aconitase
acn	gene encoding aconitase
ACS	acetyl-CoA synthase
acs	gene encoding acetyl-CoA synthase
AC_{SPE}	aqueous condensate after solid phase extraction
A. niger	*Aspergillus niger*
ADP	adenosine diphosphate
AF	mixture of acetate and formate
AFP	mixture of acetate, formate and propionate
AP	mixture of acetate and propionate
ATP	adenosine triphosphate
B. licheniformis	*Bacillus licheniformis*
B. subtilis	*Bacillus subtilis*
BM	biomass
C. glutamicum	*Corynebacterium glutamicum*
C. basilensis	*Cupriavidus basilensis*
C. necator	*Cupriavidus necator*
CS	carbon source
DECHEMA	Deutsche Gesellschaft für chemische Apparatewesen, Chemische Technik und Biotechnologie
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)

E. coli	*Escherichia coli*
e.g.	lat. exempli gratia (for example)
EDTA	ethylenediaminetetraacetic acid
et al.	lat. et aliae (and others)
F	formate
$FADH_2$	flavin adenine dinucleotide (reduced)
FDH	formate dehydrogenase
FTL	formate-tetrahydrofolate ligase
GC-MS	gas chromatoraphy - mass spectrometry
GTP	guanosine triphosphate
HB	hydroxybutyrate
HMF	5-hydroxy-2-methylfurfural
HV	hydroxyvalerate
ICL	isocitrate lyase
LGK	levoglucosan kinase
lgk	gene encoding levoglucosan kinase
IKFT	Institut für Katalyseforschung und -technologie (Institute of Catalysis Research and Technology)
KIT	Karlsruhe Institute of Technology
LB	lysogeny broth
MCC	methylcitrate cycle
MCD	methylcitrate dehydratase
MCL	2-methylcitrate lyase
MCS	methylcitrate synthase
MO	microorganism
MS	malate synthase
MWK	Ministerium für Wissenschaft, Forschung und Kunst (Ministry of Science, Research and the Arts)
n.a.	not applicable / not available
n.d.	not determined / not detectable
n.q.	not quantifiable compound
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
OC	organic condensate
OC_{FANC}	organic condensate after filtration, autoclaving, neutralization and centrifugation
OC_{SPE}	organic condensate after solid phase extraction
OD_{600}	optical density at a wavelength of 600 nm

P	propionate
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
PCS	propionyl-CoA synthase
PEP	phosphoenolpyruvate
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate
prpB	gene encoding 2-methylcitrate lyase
prpC	gene encoding methylcitrate synthase
prpD	gene encoding methylcitrate dehydratase
prpE	gene encoding propionyl-CoA synthase
PTA	phosphotransacetylase
pta	gene encoding phosphotransacetylase
rhlAB	gene encoding mono-rhamnolipid
RL	Rhamnolipid
rpm	revolutions per minute
RuMP	ribose monophosphate pathway
S. cerevisiae	*Saccharomyces cerevisiae*
SNPs	single-nucleotide polymorphisms
SPE	solid phase extraction
TCA cycle	tricarboxylic acid cycle
THF	tetrahydrofolate

Symbols

α	correlation factor
c_i	concentration of component i
Δ	delta
Ø	diameter
x g	gravitational acceleration
λ	optical wavelength
q_{RL}	rhamnolipid production rate
μ	specific growth rate
$Y_{m\|n}$	yield coefficient m to n

List of Figures

List of Tables

Appendix

Table S1: GC-MS analysis of OC and AC by Thünen Institute Hamburg

Compound	OC [wt%]	AC [wt%]
NONAROMATIC COMPOUNDS		
Acids		
Acetic acid	5.004	4.492
Propionic acid	1.302	0.404
Butyric acid	0.164	–
Nonaromatic Alcohols		
Ethylene glycol	1.258	0.437
Methanol	–	1.689
Nonaromatic Aldehydes		
Acetaldehyde, hydroxy-	0.314	–
Crotonaldehyde, trans	–	0.142
Nonaromatic Ketones		
Acetol (Hydroxypropanone)	4.631	3.484
Acetonylacetone (Hexandione, 2,5-)	0.039	–
Butanone, 2-	0.038	–
Butanone, 1-hydroxy-2-	0.844	0.490
Butandione, 2,3- (Diacetyl)	0.036	0.430
Acetoin (hydroxy-2-butanone, 3-)	0.119	0.095
Propanone, acetyloxy-2-	0.218	0.125
Cyclopentanone	0.095	0.074
Cyclopenten-1-one, 2-	0.308	0.262
Cyclopenten-1-one, 2,3-dimethyl-2-	0.222	0.045
Cyclopenten-1-one, 2-methyl-2-	0.117	0.125
Cyclopenten-1-one, 3-methyl-2-	0.229	0.058
Cyclopenten-3-one, 2-hydroxy-1-methyl-1-	0.939	0.093
Cyclopenten-1-one, 3-ethyl-2-hydroxy-2-	0.300	0.021
Cyclohexen-1-one, 2-	0.024	–

Compound	**OC [wt%]**	**AC [wt%]**
CARBOHYDRATES		
Sugars		
Levoglucosan	0.965	–
HETEROCYCLIC COMPOUNDS		
Furans		
Furfuryl alcohol, 2-	0.124	0.020
Furanone, 2(5H)-	0.176	0.027
Furfuryl alcohol, 2-	0.124	0.020
Furaldehyde, 2-	0.265	0.281
Furaldehyde, 3-	0.026	0.057
Furaldehyde, 5-methyl-2-	0.032	0.016
Ethanone, 1-(2-furanyl)-	0.041	0.043
Furan-2-one, 3-methyl-, (5H)-	0.094	0.024
Butyrolacetone, y-	0.335	0.115
AROMATIC COMPOUNDS		
Benzenes		
Benzofuran, 2-methyl-	0.016	–
Inden-1-one, 2,3-dihydro-1H-	0.044	–
Catechols	*n.q.*	*n.q.*
Aromatic Ketones		
Acetophenone	0.017	0.010
Lignin derived Phenols		
Phenol	0.384	0.041
Cresol, o-	0.151	0.031
Cresol, p-	0.134	0.015
Cresol, m-	0.170	0.012
Phenol, 2,5-dimethyl-	0.068	–
Phenol, 2,4-dimethyl-	0.053	–
Phenol, 2,6-dimethyl-	0.047	0.005
Phenol, 2,3-dimethyl-	0.030	–
Phenol, 3,5-dimethyl-	0.030	–
Phenol, 2,4,6-trimethyl-	0.012	–
Phenol, 2-ethyl-	0.065	–
Phenol, 3-ethyl-	0.067	–
Phenol, 4-ethyl-	0.171	0.008

Compound	**OC [wt%]**	**AC [wt%]**
Guaiacols (Methoxy phenols)		
Guaiacol	0.469	0.104
Guaiacol, 4-methyl-	0.150	0.019
Guaiacol, 4-ethyl-	0.191	0.009
Guaiacol, 4-allyl- (Eugenol)	0.085	–
Guaiacol, 4-propyl-	0.039	–
Guaiacol, 4-propenyl-cis (Isoeugenol)	0.144	–
Guaiacol, 4-propenyl-trans (Isoeugenol)	0.524	–
Vanillin	0.200	–
Guaiacyl acetone	0.094	–
Coniferylaldehyde	0.023	–
Syringols (Dimethoxy phenols)		
Syringol	0.556	0.011
Syringol, 4-methyl-	0.152	–
Syringol, 4-ethyl-	0.094	–
Syringol, 4-allyl-	0.079	–
Syringol, 4-propyl-	0.048	–
Syringaldehyde	0.177	–
Acetosyringone	0.095	–

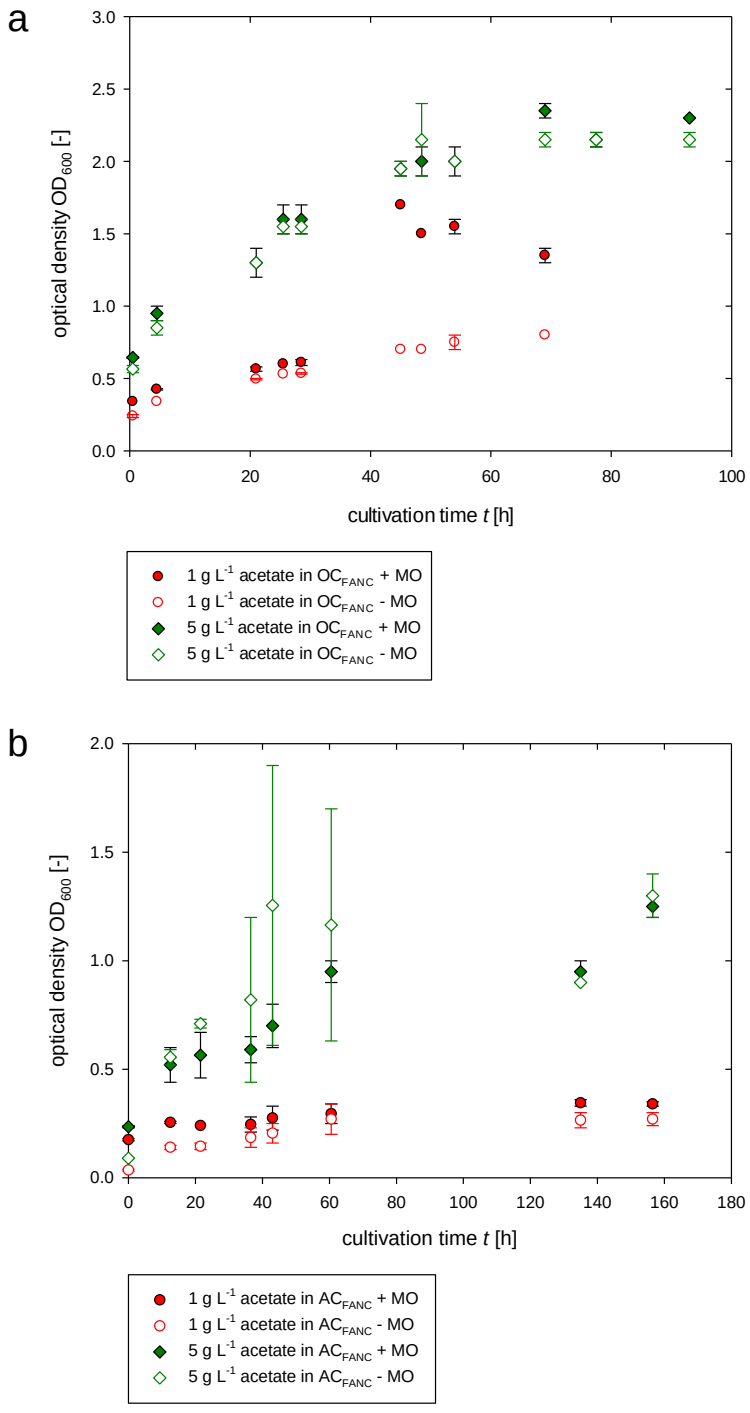

Figure S1: Supporting information to **Part I** Figure 4c and 4d – Time course of the optical density OD_{600} of OC_{FANC} or AC_{FANC} with and without microorganisms